I. Preliminary Remarks and Amendments

Applicants thank Examiner Prema Mertz for her time in discussing this application and the pending Office Action on January 16, 2007 with William Merkel and Lynn Janulis, agents for Applicants.

Claims 1, 4-8, 10, 51-55, 70, 72, and 73 are currently pending and are under examination in the present application. Claim 73 is cancelled herein. New claims 74-75 have been added. Support for new claim 74 is found throughout the specification, including at page 75, lines 18-23, and page 114, lines 5-16. Support for new claim 75 is found throughout the specification, including at page 105, line 34, through page 106, line 5; and at page 107, lines 6-11. Accordingly, the amendments do not include new matter.

Applicants do not intend, with these or any other amendments, to abandon the subject matter of claims previously presented, and reserve the right to pursue such subject matter in duly filed continuing patent applications.

II. Patentability Arguments

A. The Rejection of Claims 1, 4-8, 10, 51-55, 70, 72, and 73 under 35 U.S.C. § 101 May Properly Be Withdrawn.

The Examiner rejected claims 1, 4-8, 10, 51-55, 70, 72, and 73 under 35 U.S.C. § 101 as assertedly not being supported by either a specific and substantial utility or a well-established utility. Office Action at pages 2-7. In response, Applicants respectfully traverse.

In the Office Action, the Examiner supported the rejection by asserting that the identification of agp-96614-a1 (the nucleotide sequence set forth in SEQ ID NO: 1) in human testis, although indeed novel and unobvious, is not patentable because the product does not fulfill the utility requirement for patentability. The Examiner asserted that one of skill in the art would not recognize a specific and substantial biological role for a novel testis-specific protein, which is preferentially expressed in testis cells, without a well-established or disclosed correlation or relationship between the claimed polypeptide and a disease or disorder. *See* Office Action at pages 2-3. The Examiner further argued that one needs to

know if the claimed polynucleotide is present exclusively in cancer tissue or is overexpressed in diseased tissue compared to normal tissue. *Id.* at pages 3-4. The Examiner also took the position that PSA is useful in the diagnostics and monitoring of treatment of prostatic disease because PSA is exclusive to the prostate, and there is no exclusive or organ-specific expression of the claimed nucleic acid. *Id.* at pages 4-5. Moreover, the Examiner has taken the position that Applicants are arguing exclusive and tissue-specific expression in the testis when the specification says otherwise. *Id.* at pages 5-6. The Examiner also asserted that there is no disclosure for the use of the claimed subject matter as a tissue-specific marker in the application as filed. *Id.* at page 6. The Examiner concluded by stating that because the utility of the claimed nucleic acid is not asserted in the specification, and because it is not specific or substantial, one of skill would have to conduct further experiments to determine the particular biological functions of the claimed nucleic acid. *Id.* at pages 6-7. Applicants address each of the Examiner's arguments in the following remarks.

To satisfy the requirements of 35 U.S.C. § 101, an applicant must claim an invention that is statutory subject matter and must show that the claimed invention is "useful" for some purpose either explicitly or **implicitly**." M.P.E.P. § 2107.01 (emphasis added). Furthermore, an invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., **properties** or applications of a product or process), and (ii) the utility is specific, substantial, and credible. M.P.E.P. § 2107.01 (emphasis added). If an invention has a well-established utility, rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, based on lack of utility should not be imposed. *In re Folkers*, 344 F.2d 970 (CCPA 1965). M.P.E.P § 2107.02 further provides an example that if an application teaches the cloning and characterization of the nucleotide sequence of a well-known protein, such as insulin, and those skilled in the art at the time of filing knew that insulin had a well-established use, it would be improper to reject the claimed invention as lacking utility solely because of the omitted statement of specific and substantial utility.

In response to the Examiner's assertion that one of skill in the art would not recognize a specific and substantial biological role for a novel testis-specific protein, which is preferentially expressed in testis cells, without a well-established or disclosed correlation or relationship between the claimed polypeptide and a disease or disorder, Applicants

respectfully disagree. A person having ordinary skill in the art would have recognized a specific and substantial biological role for a novel testis-specific protein. As Applicants have previously established, a testis-specific protein is useful as a tissue-specific marker for detecting testis cells which have moved outside of the testis, either as a micrometastatic deposit of cells into the lymph nodes or bone marrow, or as a secretion into the bloodstream to indicate the presence of an abnormal condition of the testis, such as metastasized testicular cancer. Tissue-specific markers are not specifically related to cancer necessarily, but elevated levels of a tissue-specific mRNA or protein are useful for identifying a specific tissue as being at fault or suspect. For example, as provided previously, prostate-specific antigen (PSA) is a protein which is preferentially expressed in the prostate and has proven to be a very useful human tissue-specific marker for detecting prostate abnormalities, either as an indicator of prostate cells which have moved outside of the prostate as a tumor (i.e., metastatic prostate cancer) or as an elevated level of PSA in the bloodstream, which can indicate the presence of prostate cancer or benign prostatic hyperplasia (Mulders et al., *Eur. J. Surg. Oncol.* 16:37-41, 1990 (already of record)). Moreover, PSA, like the protein encoded by the claimed nucleic acid, is not exclusively expressed in one tissue. PSA, although expressed predominantly in the prostate, has been found to be expressed in other organs (Ishikawa, *Jpn. J. Clin. Oncol.* 28:723-728, 1998; Bodey et al., *Anticancer Res.* 17:2343-2346, 1997; Bodey et al., *Anticancer Res.* 17:2577-2582, 1997; *see* **Exhibit A**). Thus, PSA is analogous to the instant situation where predominant expression is found in the testis by Northern blot analysis, but expression in other tissues (e.g., pancreas and two cancer cell lines) was found by RT-PCR. It is well-known in the art that multiple markers are often used in the diagnosis of disease (Kuriyama et al., *JNCI* 68:99-105, 1982; *see* **Exhibit A**).

In response to the Examiner's assertion that one needs to know if the claimed polynucleotide is present exclusively in cancer tissue or is overexpressed in diseased tissue compared to normal tissue, Applicants respectfully disagree. Applicants respectfully submit that differential expression does not need to be shown to establish the utility of agp-96614-a1 as a marker for testicular cells. A tissue-specific marker is useful in simply signaling that the testicular cell or protein is being disseminated outside of the testis. The marker's usefulness is not solely based on differential expression or exclusive expression in cancer tissue. In fact, Applicants' assertion of utility of agp-96614-a1 as a marker for disseminated testicular cells is

not dependent on a differential level of expression of agp-96614-a1 in cancerous *versus* healthy cells of the testis. The marker, agp-96614-a1, is useful for its ability to identify a testicular cell. Identification of testicular cells in a body location other than in the testis would be recognized by one of ordinary skill in the art as the identification of a cancerous testicular cell that had metastasized. Accordingly, the asserted utility does not require a detectable difference in expression in cancerous *versus* healthy testicular cells and there is, therefore, no need for comparative studies of cancerous and healthy testicular cells to support that utility.

Further, in response to the Examiner's position that PSA is useful in the diagnostics and monitoring of treatment of prostatic disease because PSA is exclusive to the prostate, and there is no exclusive or organ-specific expression of the claimed nucleic acid, Applicants again disagree. As set out above, PSA is not exclusive to the prostate. PSA is predominantly expressed in the prostate, but it is also found to be present in the pancreas and in various cancer cell lines (as is the case with agp-96614-a1 in the present invention) (Ishikawa, *supra*; Bodey et al., *supra*). Thus, agp-96614-a1, like PSA, is useful for the purpose of identifying a cell from the testis, and the expression of the protein need not be exclusive to only one tissue in order to be useful.

In response to the Examiner's position that Applicants are arguing exclusive and tissue-specific expression in the testis when the specification says otherwise, the Applicants respectfully disagree. Applicants have argued that the predominant expression of the claimed nucleic acid in human testis provides a distinguishably detectable level of expression in testis regardless of any expression in pancreas or carcinoma cell lines. Moreover, Applicants submit that the expression found in pancreas and in carcinoma cell lines was determined using the more sensitive technique of RT-PCR. In contrast, the statement in the application that expression was predominantly found in human testis was based on Northern blot data. The two statements regarding expression are not inconsistent – there is a detectable level of expression in pancreas (and in two *ex vivo* cell lines), but the predominant expression is found in human testis. Thus, the Examiner's reliance on expression in pancreas and two cell lines to undermine the express disclosure of a predominant expression in human testis is misplaced.

In response to the Examiner's assertion that there is no disclosure for the use of the claimed subject matter as a tissue-specific marker in the application-as-filed, the Applicants respectfully disagree. The application-as-filed provided experimental evidence establishing an elevated level of expression of a novel human gene, agp-96614-a1, (CD20/IgE receptor-like mRNA; SEQ ID NO: 1) in the human testis after performing Northern blot and RT-PCR analyses. *See* specification, Example 3 at page 112. The present application provides an alignment of the amino acid sequence of the polypeptide (SEQ ID NO: 2) encoded by this gene with other members in the CD20 family (*see* Figure 3). Thus, one of skill in the art would recognize that Applicants identified a novel testis-specific form of a CD20/IgE receptor-like molecule. The agp-96614-a1 protein is shown to be 200 amino acids, and other members of this family of proteins have been shown to contain four putative transmembrane regions, N- and C-terminal cytoplasmic domains, and three inter-transmembrane loop regions. In fact, three later publications identified the exact same molecule (i.e., agp-96614-a1) as being a testis-expressed transmembrane 4 protein (Ishibashi et al., *Gene* 264:87-93, 2001; Liang et al., *Genomics* 72:119-127, 2001; Hulett et al., *Biochem. Biophys. Res. Commun.* 280:374-379, 2001; *see* Appendix C of the response filed January 15, 2003). Thus, the application disclosed the complete structures of a protein and encoding nucleic acid and showed preferentially elevated expression in testis cells.

Moreover, the Patent Office's own Utility Guidelines (at page 7) provides that "well-established utility" is a specific, substantial, and credible utility which is well known, immediately apparent, or implied by the specification's disclosure of the properties of a material, alone or taken with the knowledge of one skilled in the art. Applicants submit that one skilled in the art would have readily recognized the utility of agp-96614-a1 as a testicular cell marker after reading the specification. At minimum, there is an implicit disclosure of the use of the claimed subject matter as a marker for testicular cell dissemination or metastasis, and such a disclosure is sufficient under 35 U.S.C. §101. As stated in M.P.E.P. §2107.03, "a reasonable correlation between the evidence and the asserted utility is sufficient to prove utility." Applicants submit that they have established this reasonable correlation (*see*, e.g., articles in scientific journals made of record and argument provided herein and in previous responses of record).

Last, the Examiner concluded that because the utility of the claimed nucleic acid is not asserted in the specification, and because it is not specific or substantial, one of skill would have to conduct further experiments to determine the particular biological functions of the claimed nucleic acid. Applicants respectfully maintain that this position is irrelevant to the issue of satisfying the requirements of 35 U.S.C. § 101. Regardless of the particular biological function of the claimed nucleic acid, Applicants maintain that the claimed nucleic acid is useful based upon its expression pattern. Because the claimed nucleic acid is predominantly expressed in the testis, it is useful as a marker for a testicular cell or a testis-specific protein that has spread to other parts of the body (i.e., spread beyond the testis). Like PSA, whose biological function in the prostate has yet to be elucidated, agp-96614-a1 is useful simply because of the diagnostic information revealed by its expression pattern in tissues of the human body.

For all of the foregoing reasons, the Examiner has not established that the use of the claimed subject matter as a marker for testicular cells, especially for metastasized testicular cancer cells, is not specific or substantial or well established. Thus, the specification provides a well established utility that is credible, substantial, and specific. Accordingly, the rejection of all pending claims under 35 U.S.C. § 101 for an asserted lack of utility should be withdrawn.

B. The Rejection of Claims 1, 4-8, 10, 51-55, 70, 72, and 73 under 35 U.S.C. § 112, First Paragraph, May Properly Be Withdrawn.

The Examiner also rejected claims 1, 4-8, 10, 51-55, 70, 72, and 73 under 35 U.S.C. § 112, first paragraph, for lack of enablement based on the asserted lack of utility. *See* Office Action at page 7. The basis for the rejection is defective in relying on an asserted lack of patentable utility, as established above. For that reason, the rejection of claims 1, 4-8, 10, 51-55, 70, 72, and 73 under 35 U.S.C. § 112, first paragraph, for lack of enablement, has been overcome and should be withdrawn.

C. The Rejection of Claim 73 under 35 U.S.C. § 112, Second Paragraph, May Properly Be Withdrawn.

The Examiner rejected claim 73 under 35 U.S.C. § 112, second paragraph, for indefiniteness based on the asserted lack of clarity in the term "agp-96614" and for failing to recite that the nucleic acid is labeled. *See* Office Action at pages 7-8. The cancellation of claim 73 herein renders moot the rejection and it may properly be withdrawn.

CONCLUSION

In view of the preceding remarks, Applicants submit that claims 1, 4-8, 10, 51-55, 70, 72, and 74-75 are in condition for allowance. Expedited notification thereof is respectfully requested.

Dated: March 23, 2007

Respectfully submitted,

By Lynn L. Janulis

Lynn L. Janulis
Registration No.: 53,066
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Agent for Applicants

Jpn J Clin Oncol 1998;28(12)723–728

Original Articles

Expression of α-Fetoprotein and Prostate-specific Antigen Genes in Several Tissues and Detection of mRNAs in Normal Circulating Blood by Reverse Transcriptase-Polymerase Chain Reaction

Tomoyoshi Ishikawa[1], Hironobu Kashiwagi[1], Yoko Iwakami[1], Misako Hirai[1], Tomonori Kawamura[1], Yuji Aiyoshi[2], Toru Yashiro[2], Yoshihiro Ami[3], Kazuhiko Uchida[1] and Masanao Miwa[1]

[1]Department of Biochemistry and Molecular Oncology, Institute of Basic Medical Sciences and Center for Tsukuba Advanced Research Alliance, [2]Department of Surgery, Institute of Clinical Medicine, University of Tsukuba and [3]Department of Urology, Tsukuba Gakuen Hospital, Tsukuba, Ibaraki, Japan

Background: α-Fetoprotein (AFP) and prostate-specific antigen (PSA) in serum are widely used as tumor markers in the evaluation of prognosis and management of patients with hepatocellular carcinoma and prostate cancer, respectively. To establish the molecular diagnosis of cancer, reverse transcriptase polymerase chain reaction (RT-PCR) for *AFP* and *PSA* was used to identify circulating cancer cells in the blood of cancer patients. Here, we examined the tissue-specificity of *AFP* and *PSA* and tested whether *AFP* and *PSA* are suitable targets in the detection of certain cancer cells by RT-PCR using peripheral blood samples.
Methods: Tissue specificity of *AFP* and *PSA* was analyzed by Northern blotting and RT-PCR. Probes for *AFP* and *PSA* were hybridized with poly A^+ RNAs from 50 human tissues. RT-PCR for *AFP* and *PSA* mRNA was performed using several cancerous tissues and normal tissues and peripheral blood cells from seven healthy volunteers.
Results: Broad expression of *AFP* was observed in several tissues and a large amount of *AFP* mRNA was found in fetal liver. *PSA* was expressed in prostate, salivary gland, pancreas and uterus. By RT-PCR, *AFP* and *PSA* mRNA were detected in several tumors, including salivary pleomorphic adenoma, hilar bile duct carcinoma, pancreatic carcinoma, transitional cell carcinoma of urinary bladder and thyroid papillary carcinoma. Furthermore, *AFP* and *PSA* mRNAs were frequently detected by RT-PCR, even in peripheral blood cells from healthy volunteers.
Conclusions: Neither *AFP* nor *PSA* showed tissue-specific expression. *AFP* and *PSA* mRNA were detected in several diseased and non-diseased tissues and normal circulating blood by RT-PCR.

Key words: tissue-specific expression – α-fetoprotein – prostate-specific antigen – reverse transcriptase polymerase chain reaction – molecular diagnosis

INTRODUCTION

In Japan, about 300 000 people die yearly of cancer, making this disease the leading cause of death in adults (1). Non-invasive and highly sensitive diagnostic methods against cancer will undoubtedly have a major impact on cancer diagnosis and therapy. α-Fetoprotein (AFP) and prostate-specific antigen (PSA) in serum are valuable biological markers for the diagnosis, prognosis and management of patients with hepatocellular carcinoma (HCC) and prostate cancer, respectively (2,3). AFP is a serum protein mainly synthesized in mammalian fetal liver cells (4–8). PSA is a glycoprotein (9,10) up-regulated by androgenic hormones at the transcriptional level (11) and mainly produced in the prostate. Recently, the detection of circulating cancer cells by reverse transcriptase-polymerase chain reaction (RT-PCR) for the evaluation of post-operative prognosis, molecular staging of cancers (12) and diagnosis of micro-metastasis (13) has been reported. In these reports, tumor-specific mRNA has been

Received July 13, 1998; accepted August 31, 1998

For reprints and all correspondence: Kazuhiko Uchida, Department of Biochemistry and Molecular Oncology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: kzuchida@md.tsukuba.ac.jp

Abbreviations: AFP, α-fetoprotein; PSA, prostate-specific antigen; RT-PCR, reverse transcriptase polymerase chain reaction; HCC, hepatocellular carcinoma; AGPC, acid guanidinium thiocyanate–phenol–chloroform; MuLV, murine leukemia virus

employed to detect circulating cancer cells in peripheral blood cells of cancer patients. Detection of *AFP* and *PSA* mRNA by RT-PCR in peripheral blood has become one of the most useful molecular biomarkers (12,14–20) in cancer diagnosis. PCR is an extremely sensitive method to detect even small amounts of DNA/RNA sequences. Because of its high sensitivity, false-positive results can possibly occur even if *AFP* and *PSA* mRNA are used as targets for RT-PCR. Recent studies have reported detection of *PSA* mRNA by RT-PCR in several cancers other than prostate cancer (21–25). Expression of *AFP* in normal renal cells has also been detected (26). Low levels of *PSA* and *AFP* transcripts in peripheral blood from non-cancer patients can possibly affect the ability of RT-PCR to detect circulating cancer cells. Tissue specificity of gene expression of *PSA* and *AFP* is crucial in the establishment of cancer diagnosis by RT-PCR using peripheral blood.

In this study, we analyzed the tissue specificity of *AFP* and *PSA* in 43 human adult and seven fetal tissues by Northern blotting and examined the expression of *AFP* and *PSA* by RT-PCR using several tumors and normal peripheral blood cells.

MATERIALS AND METHODS

Probes

cDNA probes for *AFP* and *PSA* mRNAs were prepared by RT-PCR using total RNA from normal liver and prostate, respectively. The sequences of *AFP* primers were 5-GTT GCC AAC TCA GTG AGG AC-3 for the forward primer (AFP-F) and 5-GAG CTT GGC ACA GAT CCT TA-3 for the reverse primer (AFP-R). The sequences of *PSA* primers were 5-CCC ACA CCC GCT CTA CGA TA-3 for the forward primer (PSA-F) and 5-ACC TTC TGA GGG TGA ACT TGC G-3 for the reverse primer (PSA-R). The PCR products of *AFP* and *PSA* cDNAs are 240 and 289 base pairs in length, respectively. The RT-PCR products of *AFP* and *PSA* were purified with a MERmaid Kit (BIO 101, Vista, CA) and subcloned into pCR 2.1 (Original TA Cloning Kit, Invitrogen, San Diego, CA). The resulting plasmids were digested by *Eco*RI and the inserts were electrophoretically separated on a 0.8% agarose gel for purification. Human ***ubiquitin*** cDNA (CLONTECH, Palo Alto, CA) was used for standardization. The isolated DNA fragments were radiolabeled with [α-^{32}P]dCTP using a Multiprime DNA Labeling System (Amersham, UK). Plasmid inserts were sequenced by the dye primer method using a DNA Sequencing Kit with Dye Primer Cycle Sequencing Ready Reaction (Perkin-Elmer-Cetus, Foster City, CA) with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer-Cetus).

Northern Blotting

Tissue specificity was analyzed by using a Human RNA Master Blotting (CLONTECH). Hybridization was performed according to the manufacturer's protocol. Amounts of 20 ng of probes were labeled with [α-^{32}P]dCTP and then added to a mixture of 25 µg of human Cot-1 DNA (GIBCO BRL, Rockville, MD), 125 µg of sheared salmon testis DNA and 5 ml of ExpressHyb solution (CLONTECH). The blot was incubated at 65°C for 16 h. After washing four times with 2× SSC and 1% SDS at 65°C for 20 min, two additional 20 min washes were performed in 0.1× SSC and 0.5% SDS at 65°C. The damp blot was exposed on an Imaging Plate (FujiPhoto Film, Tokyo). Dots on the autoradiograph were analyzed and quantified with a BAS 5000 Imaging Analyzer (FujiPhoto Film). To determine the tissue specificity of the gene expression, the intensity percentage (% intensity) of each dot was calculated as follows according to the manufacturer's protocol. The raw data of the position of yeast transfer RNA on the blot (H2) was set as the background of the blot (data not shown in Figs 1 and 2). Signal intensity values for each dot were divided by the product of the original mRNA amounts (ng) and the scan area (mm^2). The value thus obtained in certain tissues was divided by the sum of all obtained values of the blotting and shown as % intensity. The ratio of the expressed mRNA in each tissue was obtained from this calculation.

RNA Preparation and RT-PCR

Total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform extraction (AGPC) method (27) from the following tissues: normal liver, hilar bile duct carcinoma, prostate cancer, benign prostatic hypertrophy, normal prostate, pancreatic carcinoma, normal pancreas, pleomorphic adenoma of parotid gland, normal parotid gland, bladder cancer, thyroid papillary carcinoma and normal thyroid gland. Whole blood was subjected to a Ficcoll–Conray gradient (IBL, Fujioka) and the nucleate cells were collected. Total RNAs from 5 ml of peripheral whole blood of seven healthy volunteers were also extracted by the AGPC method. Total RNA was finally dissolved in diethylpyrocarbonate-treated water to a final concentration of 1 µg/µl. Prior to reverse transcription, the total RNA solution was heated at 70°C for 10 min and then immediately placed on ice for 5 min. cDNA was synthesized using a RNA PCR Core Kit (Perkin-Elmer-Cetus) from 1 µg of total RNA in 20 µl of reaction mixture containing 1× PCR Buffer II, 1 mM dNTP, 2.5 mM oligo-d(T)$_{16}$, 20 units of RNasin and 50 units of murine leukemia virus (MuLV) reverse transcriptase. Reverse transcription was carried out at 42°C for 15 min. A 5 µl volume of the reaction mixture was used for PCR. PCR was carried out in 50 µl of reaction mixture containing 1× PCR Buffer II containing AmpliTaq Gold (Perkin-Elmer-Cetus) DNA polymerase. To obtain the most effective amplification by AmpliTaq Gold DNA polymerase, the PCR cycle was increased up to 50 cycles according to the manufacturer's protocol. PCR cycles for *AFP* and *PSA* were as follows: initial denaturation at 95°C for 10 min, followed by 50 cycles of 94°C for 1 min, 58°C for 2 min, 72°C for 3 min and final extension at 72°C for 7 min. RT-PCR of β-*actin* was used as an internal control as described (28). The sequences of β-*actin* primers are 5-AGA GAT GGC CAC GGC TGC TT-3 for the forward primer in exon 4 and 5-ATT TGC GGT GGA CGA TGG AG-3 for the reverse primer in exon 6. The PCR product is 406 bp in length. After reverse transcription, amplification was performed with 0.2 U of AmpliTaq DNA polymerase (Perkin-Elmer-Cetus) in 2.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM

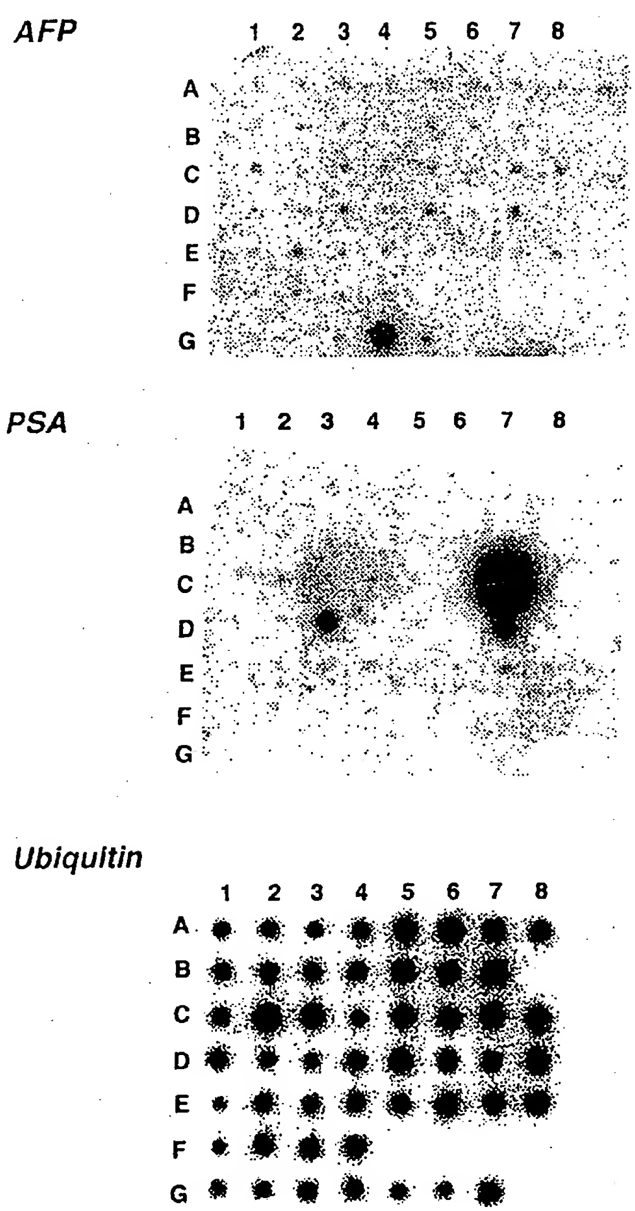


Figure 1. Expression of *AFP* and *PSA* genes in various tissues. *AFP* and *PSA* cDNAs were hybridized with poly A+ RNAs from 50 tissues. Hybridization of *ubiquitin* cDNA was performed for standardization. *AFP* was expressed in more than 30 types of tissue. A strong signal was observed in fetal liver. *PSA* was expressed mainly in prostate, salivary gland and pancreas. The type and position of poly A+ RNAs on the blot are as follows: A1, whole brain; A2, amygdala; A3, caudate nucleus; A4, cerebellum; A5, cerebral cortex; A6, frontal lobe; A7, hipocampus; A8, medulla oblongata; B1, occipital lobe; B2, putamen; B3, substantia nigra; B4, temporal lobe; B5, thalamus; B6, subthalamic nucleus; B7, spinal cord; C1, heart; C2, aorta; C3, skeletal muscle; C4, colon; C5, bladder; C6, uterus; C7, prostate; C8, stomach; D1, testis; D2, ovary; D3, pancreas; D4, pituitary gland; D5, adrenal gland; D6, thyroid gland; D7, salivary gland; D8, mammary gland; E1, kidney; E2, liver; E3, small intestine; E4, spleen; E5, thymus; E6, peripheral lymphocytes; E7, lymph node; E8, bone marrow; F1, appendix; F2, lung; F3, trachea; F4, placenta; G1, fetal brain; G2, fetal heart; G3, fetal kidney; G4, fetal liver; G5, fetal spleen; G6, fetal thymus; and G7, fetal lung.

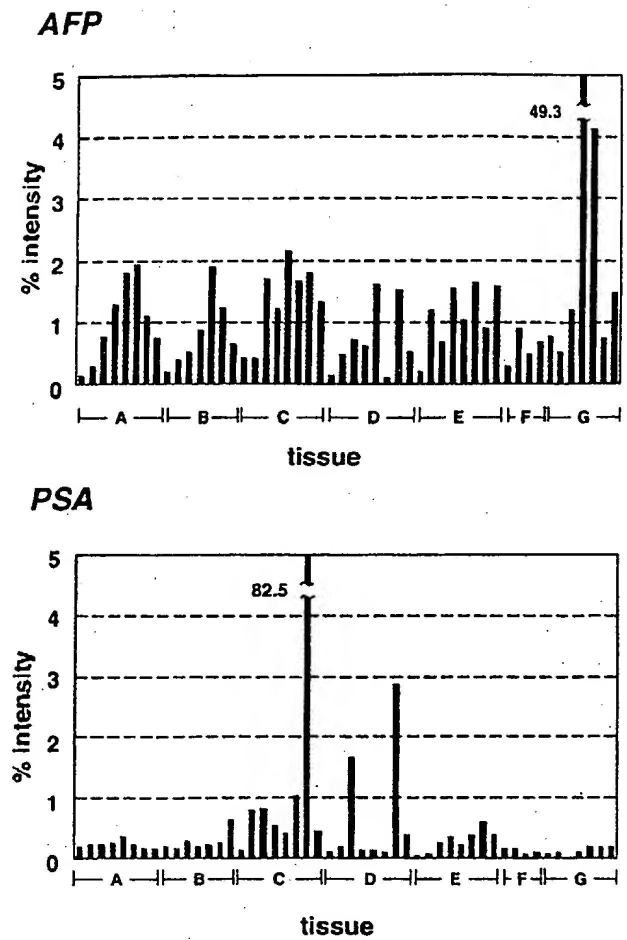


Figure 2. Intensity percentage of *AFP* and *PSA* gene expression. Each bolt was analyzed using Phosphorimager and quantified with the BAS 5000 Imaging Analyzer. The % intensity for each tissue was calculated as described in Materials and Methods. Tissue samples are indicated in groups from A to G. From the left, A1–8, B1–7, C1–8, D1–8, E1–8, F1–4 and G1–7 are shown. Definitions as in Fig. 1.

KCl, 0.01% gelatin (w/v), 0.25 mM of each dNTP and 5 pmol of each primer, by a precycle at 94°C for 3 min and subsequently 40 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C in a thermal cycler (Perkin-Elmer-Cetus). RT-PCR products of *AFP*, *PSA* and *β-actin* were separated electrophoretically on a 2.0% agarose gel and detected with ethidium bromide staining.

RESULTS

Tissue Specificity of AFP and PSA Gene Expression

Fig. 1 shows the expression of *AFP* and *PSA* genes in 50 human tissues including fetal organs. The total quantity of mRNA in each dot ranges between 95 (E6; peripheral leukocytes) and 461 ng/dot (D3; pancreas). The *AFP* gene was highly expressed in fetal liver. However, the broad expression pattern of the *AFP* gene was observed. Positive signals were found in several kinds of tissue, including peripheral leukocytes. *AFP* mRNA was also detected

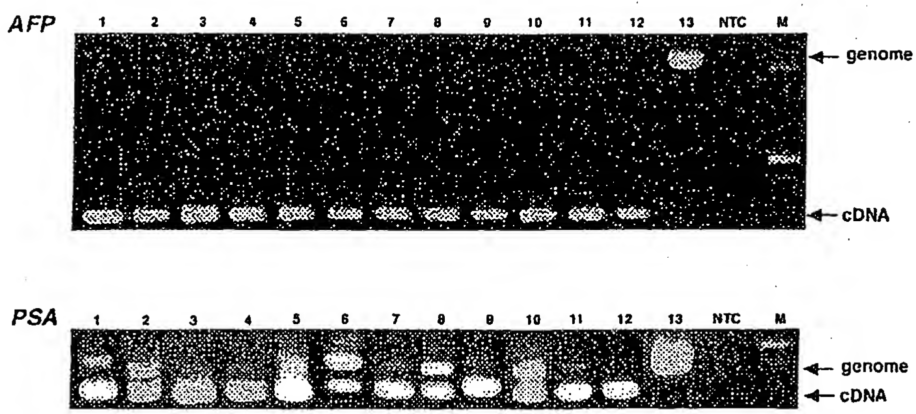


Figure 3. Detection of *AFP* and *PSA* mRNA in various cancers by RT-PCR. *AFP* and *PSA* transcripts were 240 and 289 bp, respectively. Lanes in each panel are as follows: 1, normal liver; 2, hilar bile duct carcinoma; 3, prostatic cancer; 4, benign prostatic hypertrophy; 5, normal prostate; 6, pancreatic carcinoma; 7, normal pancreas; 8, pleomorphic adenoma of parotid gland; 9, normal parotid gland; 10, bladder cancer; 11, thyroid papillary carcinoma; 12, normal thyroid gland; 13, DNA from normal lymphocytes as a negative control; NTC, no template control; M, DNA marker (pBR322 *Hin*fI digest).

in various kinds of fetal tissues. Fig. 2 shows the % intensity of expression of *AFP* and *PSA* genes to evaluate the tissue specificity of the gene expression. A high % intensity was observed for *AFP* mRNA in fetal liver (49.3%), fetal spleen (4.2%), bladder (2.1%), frontal lobe (1.9%) and thalamus (1.9%). Peripheral leukocytes showed expression of *AFP* at 1.64% intensity. Thus, *AFP* mRNA was detected in almost all tissues, including peripheral leukocytes, even at low levels.

The *PSA* gene also showed broad expression patterns as shown in Figs 1 and 2. Strong signals were observed in prostate, salivary gland, pancreas and uterus. The % intensities of *PSA* were prostate 82.5%, salivary gland 2.9%, pancreas 1.7% and uterus 1.0%. Signals for *PSA* were detected in almost all tissues, including peripheral leukocytes (0.4%). Small amounts of *PSA* mRNA were also found in various kinds of adult tissues. Fetal kidney was the only tissue where the signal for *PSA* mRNA was not detected.

Detection of *AFP* and *PSA* mRNA by RT-PCR in Cancers and Normal Peripheral Blood

We analyzed *AFP* and *PSA* expression in several cancer tissues to examine the cancer specificity of their expression. *AFP* mRNA was detected in normal liver, hilar bile duct carcinoma, prostate cancer, benign prostatic hypertrophy, normal prostate, pancreatic carcinoma, normal pancreas, pleomorphic adenoma of parotid gland, normal parotid gland, bladder cancer, thyroid papillary carcinoma and normal thyroid gland (Fig. 3). *PSA* mRNA was detected not only in prostate but also in normal liver, hilar bile duct carcinoma, pancreas carcinoma, normal pancreas, pleomorphic adenoma of parotid gland, normal parotid gland, bladder cancer, thyroid papillary carcinoma and normal thyroid gland (Fig. 3). By RT-PCR, *AFP* mRNA was detected in peripheral blood from six out of seven healthy volunteers and *PSA* mRNA was also detected in five out of seven healthy volunteers (Fig. 4). *β-actin* mRNA as internal control was detected in all cases by RT-PCR. In some cases, contaminated genomic DNA was observed at about 432 bp in PCR for *PSA*, whereas no band from genomic DNA was observed in PCR for *AFP* because of the large size (~1600 bp) of the expected PCR product from genomic DNA and the small amount of contaminated genomic DNA.

DISCUSSION

We have found that various human tissues, including peripheral leukocytes, expressed the *AFP* and *PSA* genes. mRNAs for these genes were frequently detected in normal circulating blood. Northern blot analysis using poly A$^+$ RNAs from 50 human tissues including fetal organs revealed that both *AFP* and *PSA* expression were not tissue specific. Various tissues, including peripheral blood, showed positive spots on the blot. Furthermore, RT-PCR for *AFP* and *PSA* mRNAs indicated the presence of these mRNAs in normal peripheral blood cells. These data suggest a limitation of PCR-based methods with these weak tumor- or tissue-specific mRNAs as targets. Because of the very high sensitivity of PCR, low levels of these tumor-specific transcripts, as shown by Northern blotting (Figs 1 and 2), can be detected in peripheral blood cells from non-cancer patients and healthy volunteers.

By quantification of the signals on the Northern blot, several tissues expressed *AFP* at about 1/25th of the level of the fetal liver. By RT-PCR, we found *AFP* mRNA in cancers and benign tumors such as adenoma of parotid gland, bile duct cancer, pancreas cancer, bladder cancer and thyroid cancer. *AFP* gene expression in neonatal rat kidney has been reported (26). We also showed *AFP* expression in fetal kidney.

PSA is believed to be expressed exclusively in prostatic epithelial cells. However, in the present study, expression of the *PSA* gene was observed not only in the prostate but also in various other tissues including pancreas, salivary gland and uterus as well as peripheral blood. However, the expression levels in these tissues, excluding prostate, were very low. The % intensity for

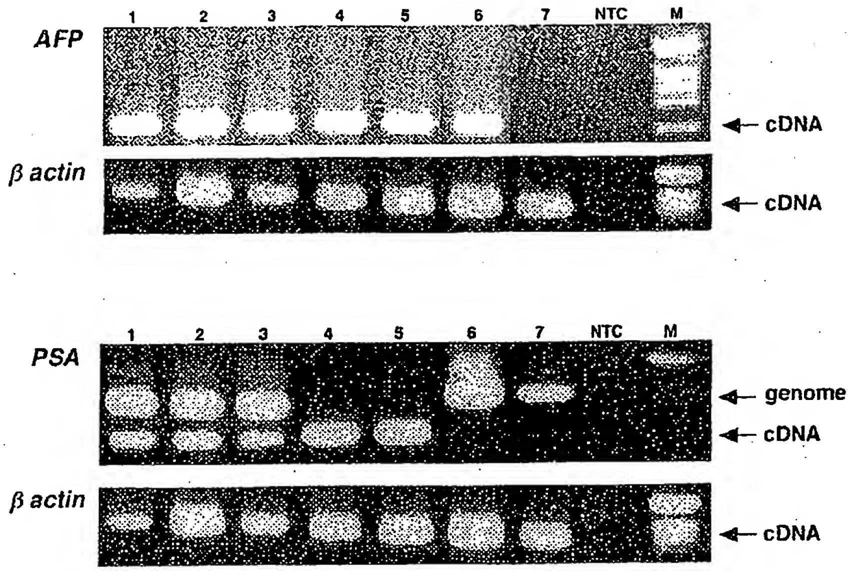


Figure 4. Detection of *AFP* and *PSA* mRNA in peripheral blood cells from healthy volunteers by RT-PCR. Lanes 1–7, peripheral blood cells from healthy volunteers; NTC, no template control; M, DNA marker (pBR322 *Hinf*I digest).

peripheral leukocytes was 0.4%, whereas prostate showed 82.5% intensity. Even at these low expression levels, we detected *PSA* mRNA in normal tissues such as liver, pancreas, parotid gland and thyroid gland and also in several cancers by RT-PCR (Figs 3 and 4). *PSA* mRNA was frequently detected in peripheral blood cells from healthy volunteers by RT-PCR. These data coincide with previous reports indicating expression of *PSA* in non-prostate cells including normal blood cells (29–32). Convincing evidence has been described for expression of *PSA* in normal tissues such as salivary gland (31), lung (33) and endometrium (34) and also in tumors such as lung cancer (23,35), breast cancer (21), ovarian tumor (24) and other tumors (25). Recent RT-PCR studies suggested expression of the *PSA* gene in breast and lung cancers (21–23,35).

The present study clearly showed the broad expression pattern of the *AFP* and *PSA* genes and no specificity for certain cancers. Detection of the circulating cancer cells by RT-PCR for cancer- and/or tissue-specific mRNA using peripheral blood from patients would be a powerful and non-invasive diagnostic method. The reliability of this method is based on the balance of sensitivity in detection and specificity for cancer. Results for the detection of *PSA* and *AFP* in peripheral blood might be reliable if the sensitivity of detection is reduced. However, the possibility of false-positive results in the highly sensitive RT-PCR must be taken into consideration. The establishment of a molecular diagnostic system to detect circulating cancer cells using RT-PCR for more tumor- and tissue-specific mRNA would have an impact on clinical cancer research.

Acknowledgments

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Sciences, Sports and Culture and from the second term Comprehensive 10-year Strategy for Cancer Control and Cancer Research from the Ministry of Health and Welfare of Japan. We thank Drs T. Todoroki, E. Ueno and H. Hara for collecting samples and for helpful discussions and Ms A. Kikukawa for helping with DNA sequencing.

References

1. Health and Welfare Statistics Association. *Kouseinoshihyou, Kokumineisei-no-Doukou* 1997:44:48–61 (in Japanese).
2. Oesterling JE. Prostate specific antigen: a critical assessment of the most useful marker for adenocarcinoma of the prostate. *J Urol* 1991:145:907–23.
3. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N Engl J Med* 1987:317:909–16.
4. Kirsch JA, Wise RW, Oliver IT. Post-albumin, a fetal-specific rat plasma protein. Purification, physicochemical and immunological studies. *Biochem J* 1967;102:763–6.
5. Ruoslahti E, Seppala M, Pikko M, Vuopio R. Studies of carcino-fetal proteins. II. Biochemical comparison of α-fetoprotein from human fetuses and patients with hepatocellular cancer. *Int. J. Cancer* 1971:8:283–8.
6. Kahan B, Levine L. The occurrence of a serum fetal α_1 protein in developing mice and murine hepatomas and teratomas. *Cancer Res* 1971;31:930–6.
7. Branch WR, Wild AE. Localization and synthesis of α-fetoprotein in the rabbit. *Z Zellforsch Mikrosk Anat* 1972;135:501–16.
8. Tamaoki T, Thomas K, Schindler I. Cell-free studies of developmental changes in synthesis of α-fetoprotein and albumin in the mouse liver. *Nature* 1974;249:269–71.

9. Wang MC, Papsidero LD, Kuriyama M, Valenzuela LA, Murphy GP, Chu TM. prostate antigen:a new potential marker for prostatic cancer. *Prostate* 1981;2:89–96.
10. Watt KWK, Lee PJ, M'Timkulu T, Chan WP, Loor R. Human prostate-specific antigen: structural and functional similarity with serine proteases. *Proc Natl Acad Sci USA* 1986;83:3166-70.
11. Henttu P, Liao S, Vihko P. Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology* 1992:130:766–72.
12. Katz AE, Seaman ES, Olsson CA, O'Toole KM, Raffo AJ, McMahon D, et al. Molecular staging of prostate cancer with the use of an enhanced reverse transcriptase-PCR assay. *Urology* 1994;43:765–75.
13. Mori M, Mimori K, Ueo H, Tsuji K, Shiraishi T, Barnard GF, et al. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. *J Clin Oncol* 1998;16:128–32.
14. Funaki N, Tanaka J, Seto S, Kasamatsu T, Kaido T, Imamura M. Highly-sensitive identification of α-fetoprotein mRNA in circulating peripheral blood of hepatocellular carcinoma patients. *Life Sci* 1995;57:1621–31.
15. Louha M, Poussin K, Ganne N, Zylberberg H, Nalpas B, Nicolet J, et al. Spontaneous and iatrogenic spreading of liver-derived cells into peripheral blood of patients with primary liver cancer. *Hepatology* 1997:26:998–1005.
16. Kawakami M, Okaneya T, Furihata K, Nishizawa O, Katsuyama T. Detection of prostate cancer cells circulating in peripheral blood by reverse transcription-PCR for *hKLK2*. *Cancer Res* 1997;57:4167–70.
17. Seiden MV, Kantoff PW, Krithivas K, Propert K, Bryant M, Haltom E, et al. Detection of circulating tumor cells in men with localized prostate cancer. *J Clin Oncol* 1994;12:2634–9.
18. Israeli RS, Miller WH, Su SL, Powell CT, Fair WR, Samadi DS, et al. Sensitive nested reverse transcriptase polymerase chain reaction detection of circulating prostate tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. *Cancer Res* 1994;54:6306–10.
19. Ennis RD, Katz AE, de Vries GM, Heitjan DF, O'Toole KM, Rubin M, et al. Detection of circulating prostate carcinoma cells via an enhanced reverse transcriptase chain reaction assay in patients with early stage prostate carcinoma. Independence from other pretreatment characteristics. *Cancer* 1997;79:2402–8.
20. Johnson PWM, Burchill SA, Selby PJ. The molecular detection of circulating tumour cells. *Br J Cancer* 1995;72:268–76.
21. Yu H, Giai M, Diamandis EP, Katsaros D, Sutherland DJA, Levesque MA, et al. Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer. *Cancer Res* 1995;55:2104–10.
22. Lehrer S, Terk M, Piccoli SP, Song HK, Lavagnini P, Luderer AA. Reverse transcriptase chain reaction for prostate-specific antigen may be a prognostic indicator in breast cancer. *Br J Cancer* 1996;74:871–3.
23. Bilgrami S, Singh NT, Shafi N, Ciesielski T. Raised prostate-specific antigen in adenocarcinoma of lung. *Lancet* 1994;344:1371–2.
24. Yu H, Diamandis EP, Levesque MA, Asa S, Monne M, Croce CM. Expression of the prostate-specific antigen gene by a primary ovarian carcinoma. *Cancer Res* 1995;55:1603–6.
25. Levèsque M, Yu H, D'Costa M, Diamandis EP. Prostate specific antigen expression by various tumors. *J Clin Lab Anal* 1995:9:123–8.
26. Gilbert JC, Chiu J-F. Tissue specific control of α-fetoprotein gene expression. *Biochem Biophys Res Commun* 1984;120:677–85.
27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987;162:156–9.
28. Hayakawa S, Saito S, Nemoto N, Chishima F, Akiyama K, Shiraishi H, et al. Expression of recombinase-activating genes (RAG-1 and 2) in human decidual mononuclear cells. *J Immunol* 1994;153:4934–9.
29. Kamoshida S, Tsutsumi Y. Extraprostatic localization of prostatic acid phosphatase and prostate-specific antigen: distribution in cloacogenic glandular epithelium and sex-dependent expression in human anal gland. *Hum Pathol* 1990;21:1108–11.
30. Iwakiri J, Grandbois K, Wehner N, Graves HCB, Stamey T. An analysis of urinary prostate specific antigen before and after radical prostatectomy: evidence for secretion of prostate specific antigen by the periurethral glands. *J Urol* 1993;149:783–6.
31. Van Krieken TH. Prostate marker immunoreactivity in salivary gland neoplasms. A rare pitfall in immunohistochemistry. *Am J Surg Pathol* 1990;17:410–4.
32. Wernert N, Albrech M, Sesterhum I, Goebbels R, Bonkhoff H, Seiz G, et al. The 'female prostate': location, morphology, immunohistochemical characteristics and significance. *Eur Urol* 1992;17:64–9.
33. Zarghami N, D'Costa M, Tsuyuki D, Asa SL, Diamandis EP. Expression of the prostate specific antigen gene by lung tissue. *Clin Cancer Res* 1997;3:1201–6.
34. Clements J, Mukhtar A. Glamdular kallikreins and prostate-specific antigen are expressed in human endometrium. *J Clin Endocrinol Metab* 1994;78:1536–9.
35. Levesque M, Yu H, D'Costa M, Tadross L, Diamandis EP. Immunoreactive prostate specific antigen in lung tumors. *J Clin Lab Anal* 1995:9:375–9.

ANTICANCER RESEARCH 17: 2343-2346 (1997)

Immunocytochemical Detection of Prostate Specific Antigen Expression in Human Primary and Metastatic Melanomas

BELA BODEY[1], BELA BODEY JR[1] and HANS E. KAISER[2]

[1]Department of Pathology, School of Medicine, University of Southern California, Los Angeles, CA, U.S.A.
[2]Department of Pathology, School of Medicine, University of Maryland, Baltimore, MD, U.S.A. and
Department of Clinical Pathology, University of Vienna, Vienna, Austria

Abstract. *Prostate-specific antigen (PSA), a 33 kD glycoprotein, was initially reported to be a tissue specific protein, detected in the seminal fluid and produced by normal and abnormal epithelial cells of the prostate gland, as well as other tissues in the human body. The expression of PSA has been described to be elevated during benign and neoplastic cell growth in the prostate, and in a number of other human malignancies. The presence and production of PSA in human primary cutaneous malignant melanomas (CMMs) and metastatic malignant melanomas (MMMs) has not been reported prior to the present study. We examined the expression of PSA employing a biotin-streptavidin based, alkaline phosphatase conjugated antigen detection technique in routine, neutral formalin fixed, paraffin-wax embedded, 3-4 μm thick tissue sections of 30 CMMs and 10 MMMs. Human postnatal thymic tissue, among others, was used as a negative tissue control, while normal prostate and prostate carcinomas (PCs) were included in the collection of antigen positive tissues. We observed the presence of PSA in 16/30 CMMs and 6/10 MMMs. The intensity of the staining was moderate (C to B) and localized to between 20% and 30% of the total tumor cell population in both CMMs and MMMs, with cells of similar immunoreactivity being clustered in groups within the tumor microenvironment. This result directly contradicts the previous opinion concerning the prostate epithelium specificity of PSA expression and production. The immunophenotype (IP) heterogeneity of malignant melanoma cells is further substantiated by the pattern of their PSA immunoreactivity. The establishment of the clinical significance of these findings necessitates further in vivo and in vitro research in malignant melanomas. PSA related, novel antineoplastic immunotherapy may also be recommended in the treatment of both CMMs and MMMs.*

Correspondence to: Bela Bodey MD., PhD., 15745 Saticoy Street., Van Nuys, CA 91406, USA.

Key Words: Human primary cutaneous malignant melanoma (CMM); Melanoma progression; Metastatic malignant melanoma (MMM); Immunocytochemistry; Biotin-Streptavidin linkage; Alkaline phosphatase (AP) conjugation; Prostate Specific Antigen (PSA); Immunoreactivity; Immunophenotype (IP) heterogeneity.

Prostate-specific antigen (PSA). PSA has in recent years become established as an excellent serum marker for prostate cancer (1). The mature form of PSA is a 33 kD, 237-residue glycoprotein (consisting of 93% amino acids and 7% carbohydrates), produced by prostatic epithelial cells, breast epithelium and endometrium (2,3). PSA is a serine protease, and a member of the kallikrein gene family, located on chromosome 19 (4,5).

Elevated expression of PSA has been reported in several non-prostate human tumors, including salivary gland neoplasms (6,7); breast cancer (8-10); established breast cancer cell lines (8); lung adenocarcinoma (11); parotid, liver, adrenal, kidney, colon tumors (3); ovarian carcinomas (3,12); Skene's paraurethral glands and ducts adenocarcinoma (13,14); and adenocarcinoma of the female urethra (15). The production of a 33 kDa protein similar to seminal PSA by 30% of BCs (from 525 cytosols observed) has also been described (8). The ovarian metastases of BCs have also been observed to produce PSA. Of 99 primary ovarian cancers, only three were found to produce low amounts of PSA. Additionally, PSA expression was found to be associated with that of progesterone receptors (PRs), but not the presence of estrogen receptors (ERs).

It is necessary to point out that most of these studies were carried out using various immunoassay techniques, excluding immunohistochemistry. A recent study of ours describing the detection of heterogeneous PSA immunoreactivity within the tumor microenvironment of human BCs (16), as well as the present report are two of just a handful of reports which employed sensitive immunohistochemical methods for establishing the expression of this antigen in non-prostate cancers.

Neural crest origin and neoplastic transformation of melanocytes. During early human ontogenesis, following neural induction, the neural plate of the embryonic ectoderm folds at its lateral edges forming the neural groove. After the

fusion of the edges of the neural plate, the neural tube is formed which is lined by the neuroepithelium. The neural crest is a transient and migratory group of cells that emerge from the dorsal region of the neural tube and rapidly disperse along different pathways. Their migration terminates at many peripheral locations, where they coalesce to form the neurons and Schwann cells of the sensory and autonomic nervous systems, the chromaffn cells of the adrenal medulla, the mesenchymal tissues of the face and skull, and the melanocytes of the skin (17,18).

During the last decade malignant melanoma, the deadliest of skin cancers, has become a relatively common human cancer with 32,000 new cases registered in the United States in 1992. Moreover, the incidence of this type of cancer has risen more rapidly than that of any other (19,20). It is believed that malignant melanomas are derived from dopa-positive melanocytes of neuroectodermal origin (21). The precancerous stage of melanoma has been shown to be associated with the development of common acquired melanocytic naevi (CAMN) which are composed of cytogenetically normal melanocytes. The first sign of malignant transformation into a naevus is the development of architectural atypia. A naevus demonstrating both architectural and cytological atypia is defined as a skin lesion or dysplastic naevus (DN) (22-24). Dysplastic naevi may undergo malignant transformation into primary malignant melanomas (PM), with two stages of further tumor progression. During the radial growth phase (RGPh) the melanocytic lesions are capable of only invasion, but do not develop metastases (25). In the most malignant vertical growth phase (VGPh), de novo and in situ developed malignant melanoma cells acquire the greatest potential to disseminate. The presence of metastatic malignant melanoma (MMM) is the culmination of a complex, multistep tumor progression, defned by numerous, sequential molecular-genetic events (26,27). Advanced PMs and MMMs also acquire a multicytokine resistance to interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), oncostatin M, and IL-6, as well as a multigrowth factor independence (28-30).

In view of the literature the specific aims of the present study were to: a) examine the usefulness of highly sensitive immunohistochemical techniques to detect the presence of PSA in human primary and metastasized malignant melanoma cells; b) determine the pattern of PSA immunoreactivity within the heterogenous tumor cell microenvironment employing a highly specific anti-PSA MoAB (*i.e.* whether the immunoreactivity is homogeneous or localized to distinct cell clones of melanoma cells); and c) elaborate on the possible pathogenetic significance of PSA expression in malignant melanomas.

Materials and Methods

In this immunocytochemical screening we used exclusively formalin fixed (31). paraffin embedded tissue sections of 30 primary cutaneous and 10 metastatic melanomas. The melanoma cases were obtained following therapeutic surgery as formalin fixed, paraffin embedded histopathologic specimens from the Departments of Pathology at Saint John's Hospital and the University of California at Los Angeles Medical Centers. Paraffin-wax melanoma tissue sections (3 to 4 μm thick) were cut employing a Reichert microtome and mounted on precleaned standard histological slides.

Antibody. During this immunocytochemical observation a recently developed, mouse anti-human monoclonal antibody (MoAB) against PSA was employed (from Neomarkers, Fremont, CA 94555). Working dilution: 1:60 to 1:100. To achieve optimal immunoreactivity, tumor tissue fixation time was shortened to 4-6 hours and antigen retrieval technique was applied.

Antigen retrieval. In this immunocytochemical research project, we employed the immunohistochemical procedure of heat induced epitope retrieval (HIER) (32-34) as modified by us. Antigen retrieval required immersion of tissue sections in a Target Retrieval Solution (DAKO Corporation, Carpinteria, CA, USA) and heating in a water bath (95°C to 99°C). Unmasking of fixed antigen epitopes was also carried out by a single or combined enzymatic digestion prior to the primary antigen-antibody reaction. An increase in the quantity of detectable antigenic epitopes following HIER has been described for a number of antibodies. Immunomorphological observations also reported a significant increase in the intensity of immunostaining.

Immunoalkaline phosphatase conjugated antigen detection technique. We used the following immunoalkaline phosphatase cytochemical method, modified by us for the detection of PSA in formalin fixed, paraffin-wax embedded malignant melanoma tissues (35-37). The technique is a highly sensitive, indirect, four to six step immunocytochemical method, which combines the biotin-streptavidin based ABC-method (38) with enzyme-linked (alkaline phosphatase) immunohistochemistry. Briefly, following deparaffinization in three changes of Xylene substitute (Shandon-Lipshaw, Pittsburgh, PA, USA) for 20 to 30 minutes, rehydration was carried out employing descending dilutions of alcohol (100% to 50%) to TBS. An initial blocking step using 1% glacial acetic acid mixed with the working buffer for 10 minutes was necessary to eliminate the endogenous AP activity from the tissues. Use of levamisole solution is also described in our earlier observations. As we explained earlier (37), GAA inhibition was preferred because of the possible presence of levamisole-resistant AP iso-enzyme (39). The second blocking step was conducted with a purified mixture of proteins (Shandon-Lipshaw) from various species for 5-10 minutes to block cross-reactive antigenic epitopes. Excess serum was removed from the area surrounding the sections! The tissue sections were then incubated for 90-120 minutes with the particular primary antibody. Next, incubation with the secondary antibody, a whole goat anti-mouse IgG molecule (IgG molecule diluted by ICN Biomedicals, Inc., Aurora, OH, USA) was carried out for 20 minutes. Streptavidin conjugation was accomplished by incubation with AP conjugated streptavidin for 20 minutes (40-43). The binding of the biotinylated antibody to streptavidin complexes occurs with an extremely high affinity (10^{-19}M) (BioWhitaker, Inc., Walkersville, MD, USA). Color visualization of the primary antigen-antibody (Ag-Ab) reaction was accomplished using an alkaline phosphatase (AP) kit I (Vector Laboratories, Burlingame, CA, USA) which contains ASTR with Tris-HCl buffer at pH 8.2, added for 28-40 minutes to allow formation of a stable red precipitate from the primary Ag-Ab reaction product (various intensities). Sections were counter-stained with a dilute solution of Gill's hematoxylin (Richard-Allan, Kalamazoo, MI, USA). The tissue slides were then dehydrated in ascending concentrations of alcohol (60% to 100%) to xylene substitute (Shandon-Lipshaw), in which they were kept overnight to ensure complete morphological clearing. The stained tissue sections were

mounted using a solution specially designed for use following morphological clearing in xylene substitute (Shandon-Lipshaw).

Human tissue controls in immunohistochemistry. To accurately assess the expression of PSA in primary and metastasized malignant melanoma cells, we used positive and negative tumor tissues all contained on human multitumor checkerboard tissue slides: breast, non-small cell lung, hepatocellular, colon, prostate, ovarian carcinomas and other tissues (DAKO Corp.; Lot: 5110F) (44,45). We also stained several normal, adult human thymic, tonsil, spleen, thyroid, lung, liver, kidney, heart, pancreas, ovary, prostate, small intestine, large intestine and brain tissue sections, all included in one multitissue block (DAKO Corp.; Lot: 5935B). Additional controls for all employed tissues and MoABs included:

1) omission of the primary MoAB;
2) using only the enzymatic developer solution to detect the presence of endogenous peroxidase or alkaline phosphatase activity; and
3) use of MOPC 21 mouse myeloma IgG_1 (ICN Biomedicals, Inc.) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

Evaluation of the immunocytochemical results. Qualitative and quantitative evaluations of the percent of antigen positive cells and the intensity of immunostaining were conducted using a light microscope (Olympus America, Inc., Melville, NY, USA) counting 100-200 cells from each of five to eight distinct areas in nonnecrotic malignant melanomas and positive and negative control tissues. Artifacts were avoided, while, on the other hand, morphologically characteristic areas were sought out. The presence of melanoma cells with very heterogeneous IPs, the endothelial elements of small blood vessels, tumor infiltrating leukocytes and macrophages (the host's immunological effector and antigen presenting cells) required careful qualitative assessment. Non-vascular elements were also examined, but only morphologically distinct neoplastically transformed cells were scored!

Quantitative evaluation: (37)

(++++) over 90% of the total cell number are positive; (+++) 50% to 90% of the total cell number are positive; (++) 10% to 50% of the total cell number are positive; (+) 1% to 10% of the total cell number are positive; (±) under 1% of the total cell number are positive; (-) negative.

Qualitative evaluation: (37)

(A) very intense red staining; (B) strong red staining; (C) light red staining; (D) negative staining.

Results

We observed the expression of PSA in 16 out of 30 CMMs and 6 out of 10 MMMs. The intensity of the immunoreactivity ranged from C to B and the staining was usually restricted to the cell membrane, although in MMMs staining in the cytoplasm of neoplastically transformed melanocytes was common. 20% to 30% of the total malignant melanoma cell population reacted positively with the anti-PSA MoAB in both CMMs and MMMs. The immunoreactive malignant cells were clustered in groups within the tumor cell microenvironment and formed special tumor cell clones with a highly dedifferentiated IP. The IP heterogeneity of malignant melanoma cells, previously reported in detail by us (47) using immunocytochemistry with extensive libraries of mono- and polyclonal antibodies, is further substantiated by their PSA positivity or negativity.

Discussion

The present study represents the first concerning the expression of PSA in human malignant melanomas. In this immunocytochemical investigation, we detected PSA immunoreactivity in 16 out of 30 CMMs and 6 out of 10 MMMs. The PSA immunoreactivity was localized to 20% to 30% of the total cell population in CMMs and a similar percentage of the cells stained positively in MMMs. The division of the tumor microenvironment into PSA positive and PSA negative cell groups represents another marker of cellular IP hterogeneity in human malignant melanoma, in addition to those described by us in another communication (46). The intensity of the immunoreactivity was heterogenous, ranging from moderate to strong.

Prostate specific antigen (PSA) was proclaimed as a glycoprotein produced exclusively by prostatic epithelial tissue merely three years ago by Armbruster (47). However, there is a growing amount of experimental evidence that PSA may be present in many steroid hormone stimulated tissues other than that of the prostate (3). Using reverse transcriptase-polymerase chain reaction and DNA sequencing techniques, PSA mRNA was identified in 30% of female BCs (10,48). Employing a highly sensitive immunofluorometric assay, PSA presence has been defined in 23 of 43 diverse tumor tissue extracts. As we mentioned earlier, PSA has been identified in a number of human malignancies: 30%-40% of breast carcinomas, and in a lower percentage of tumors of the skin, parotid, salivary gland, lung, colon, liver, ovary, kidney and adrenals.

The presence of PSA in the sera of patients with renal cell carcinoma and non-Hodgkin's lymphoma of the kidney has been classified as a false-positive phenomenon by several investigators (49,50). In view of the numerous studies establishing various serum PSA levels in a number of non-prostate cancers in both men and women, and the immunohistochemical data presented by us and others, it is quite clear that PSA can no longer be considered a prostate-specific molecule. Future observations will decide the true clinico-pathological value of PSA expression in human malignancies.

Acknowledgements

The authors wish to express their gratitude for the excellent assistance of Dr. Victoria Psenko in the preparation of this manuscript.

References

1 Villoutreix BO, Getzoff ED, Griffin JH: A structural model for the prostate disease marker, human prostate-specific antigen. Protein Science 3: 2033-2044, 1994.
2 Seregni E, Botti C, Ballabio G, Bombardieri E: Biochemical

characteristics and recent biological knowledge on prostate-specific antigen. Tumori 82: 72-77, 1996.

3 Levesque M, Hu H, D'Costa M, Diamandis EP: Prostate-specific antigen expression by various tumors. J Clin Lab Anal 9: 123-128, 1995.

4 el-Shirbiny AM: Prostatic specific antigen. Advances Clin Chem 31: 99-133, 1994.

5 Lilja H: Structure, function, and regulation of the enzyme activity of prostate-specific antigen. World J Urol 11: 188-191, 1993.

6 van Krieken JH: Prostate marker immunoreactivity in salivary gland neoplasms. A rare pitfall in immunohistochemistry. Am J Surg Pathol 17: 410-414, 1993.

7 Poller D: Prostate marker immunoreactivity in salivary gland neoplasms. Am J Surg Pathol 18: 214-215, 1994.

8 Yu H, Diamandis EP, Zarghami N, Grass L: Induction of prostate specific antigen production by steroids and tamoxifen in breast cancer cell lines. Breast Cancer Res Treat 32: 291-300, 1994.

9 Diamandis EP, Yu H, Sutherland DJ: Detection of prostate-specific antigen immunoreactivity in breast tumors. Breast Cancer Res Treat 32: 301-310, 1994.

10 Monne M, Croce CM, Yu H, Diamandis EP: Molecular characterization of prostatespecific antigen messenger RNA expressed in breast tumors. Cancer Res 54: 6344-6347, 1994.

11 Bilgrami S, Singh NT, Shafi N, Ciesielski T: Raised prostate-specific antigen in adenocarcinomaoflung. Lancet 344: 1371-1372, 1994.

12 Yu H, Diamandis EP, Levesque M, Asa SL, Monne M, Croce CM: Expression of the prostate-specific antigen gene by a primary ovarian carcinoma. Cancer Res 55: 1603-1606, 1995.

13 Zaviacic M, Sidlo J, Borovsky M: Prostate specific antigen and prostate specific acid phosphatase in adenocarcinoma of Skene's paraurethral glands and ducts. Virchows Arch A Pathol Anat Histopathol 423: 503-505, 1993.

14 Dodson MK, Cliby WA, Keeney GL: Peterson MF, Podratz KC: Skene's gland adenocarcinoma with increased serum level of prostate-specific antigen. Gynecol Oncol 55: 304-307, 1994.

15 Ebisuno S, Miyai M, Nagareda T: Clear cell adenocarcinoma of the female urethra showing positive staining with antibodies to prostate-specific antigen and prostatic acid phosphatase. Urology 45: 682-685, 1995.

16 Bodey B, Siegel SE, Luck JV, Kaiser HE: Immunocytochemical detection of Prostate Specific Antigen expression in human breast carcinoma cells. In print, Anticancer Res, 1997.

17 Rawles ME: Origin of melanophores and their role in development of color patterns in vertebrates. Physiol Rev 28: 383, 1948.

18 Anderson DJ: The neural crest cell lineage problem: Neuropoiesis? Neuron 3: 1-12, 1989.

19 Legha SS: Current therapy for malignant melanoma. Semin Oncol 16: 34-44, 1989.

20 Fountain JW, Bale SJ, Housman DE, Dracopoli NC: Genetics of Melanoma. Cancer Surveys 9: 645-671, 1990.

21 Clark NH, From L, Bernadino EA, Mihm MC: The histogenesis and biological behaviour of primary human malignant melanoma of the skin. Cancer Res 29: 705-726, 1969.

22 Clark WH Jr, Elder DE, Guerry D, Epstein ME, Greene MH, van Horn M: A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Human Pathol 15: 1147-1165, 1984.

23 Poste G: Pathogenesis of metastatic disease: Implications for current therapy and for the development of new therapeutic strategies. Cancer Treat Rep 70: 183-198, 1986.

24 Duray PH, Palazzo J, Gown AM, Ohuchi N: Melanoma cell heterogeneity. Cancer 61: 2460-2468, 1988.

25 Guerry D, Synnestvedt M, Elder DE, Schultz D: Lessons from tumor progression. The invasive radial growth phase of melanoma is common, incapable of metastasis, and indolent. J Invest Dermatol 100: S342-S345, 1993.

26 Herlyn M, Clark WH, Rodeck U, Mancianti ML, Jambrosic J, Koprowski H: Biology of tumor progression in human melanocytes. Lab Invest 56: 461-474, 1987.

27 Fearon ER, Vogelstein B: A genetic model for colorectal carcinogenesis. Cell 61: 759-767, 1990.

28 Kerbel RS: Expression of multi-cytokine resistance and multigrowth factor independence in advanced stage metastatic cancer. Malignant melanoma as a paradigm. Am J Pathol 141: 519-524, 1992.

29 Lu C, Kerbel RS: Interleukin-6 undergoes transition from paracrine growth inhibitor to autocrine stimulator during human melanoma progression. J Cell Biol 120: 1281-1288, 1993.

30 Weterman MAJ, van Muijen GNP, Bloemers HPJ, Ruiter DJ: Biology of Disease. Molecular markers of melanocytic tumor progression. Lab Invest 70: 593-608, 1994.

31 Farmilo AJ, Stead RH: Fixation in immunocytochemistry. In: Handbook Immunochemical Staining Methods, Ed. Naish S.J., DAKO Corporation, 1989, pp. 2429.

32 Shi S-R, Key ME, Kalra KL: Antigen retrieval in formalin fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating oftissue sections. J Histochem Cytochem 39: 741-748, 1991.

33 Shi S-R, Cote C, Kalra KL, Taylor CR, Tandon AK: A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. J Histochem Cytochem 40: 787-792, 1992.

34 Beckstead JH: Improved antigen retrieval in formalin-fixed, paraffin-embedded tissues. Appl Immunohistochem 2: 274-281, 1994.

35 Janckila AT, Yam LT, Li C-Y: Immunoalkaline phosphatase cytochemistry. Amer J Clin Pathol 84: 476-480, 1985.

36 Cattoretti G, Berti E, Schiro R, D' Amato L, Valeggio C, Rilke F: Improved avidin-biotinperoxidase complex (ABC) staining. Histol J 20: 75-80, 1988.

37 Bodey B, Zeltzer PM, Saldivar V, Kemshead J: Immunophenotyping of childhood astrocytomas with a library of monoclonal antibodies. Int J Cancer 45: 1079-1087, 1990.

38 Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison of ABC and unlabeled antibody (PAP) procedure. J Histochem Cytochem 29: 577-581, 1981.

39 Yam LT, Janckila A.T, Epremian BE, Li C-Y: Diagnostic significance of levamisole-resistant alkaline phosphatase in cytochemistry and immunocytochemistry. Amer J Clin Pathol 91: 31-36, 1989.

40 Strasburger CJ, Amir-Zaltsman Y, Kohen F: The avidin-biotin reaction as an universal amplification system in immunoassays. Prog Clin Biol Res 285: 79-100, 1988.

41 Wilchek M, Bayer EA: Introduction to avidin-biotin technology. Methods Enzymol 184: 5-13, 1990.

42 Duhamel RC, Whitehead JS: Prevention of nonspecific binding of avidin. Methods Enzymol 184: 201-207, 1990.

43 Diamandis EP, Christopoulos TK: The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem 37: 625-636, 1991.

44 Battifora H: The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest 55: 244-248, 1986.

45 Battifora H, Mehta P: The checkerboard tissue block. An improved multitissue control block. Lab Invest 63: 722-724, 1990.

46 Bodey B, Kaiser HE, Goldfarb RH: Immunophenotypically varied cell subpopulations in primary and metastatic human melanomas. Monoclonal antibodies for diagnosis, detection of neoplastic progression and receptor directed immunotherapy. Anticancer Research, 16(1): 517-531, 1996.

47 Armbruster DA: Prostate-specific antigen: biochemistry, analytical methods, and clinical application. Clin Chem 39: 181-195, 1993.

48 Zarghami N, Diamandis EP: Detection of prostate-specific antigen mRNA and protein in breast tumors. Clin Chem 42: 361-366, 1996.

49 Rasmuson T, Grankvist K, Ljungberg B: Re: False-positive prostate specific antigen values in the sera of women with renal cell carcinoma. J Urol 151: 704-705, 1994.

50 Djavan B, Keffer JH, Molberg K, Roehrborn CG: False-positive serum prostate-specific antigen values, in a patient with non-Hodgkin's lymphoma of the kidney. Urology 45: 875-878, 1995.

Received November 12, 1996
Accepted February 12, 1997

ANTICANCER RESEARCH 17: 2577-2582 (1997)

Immunocytochemical Detection of Prostate Specific Antigen Expression in Human Breast Carcinoma Cells

BELA BODEY[1], BELA BODEY, Jr.[1] and HANS E. KAISER[2,3]

[1]*Department of Pathology, School of Medicine, University of Southern California, Los Angeles, CA;*
[2]*Department of Pathology, School of Medicine, University of Maryland, Baltimore, MD;*
[3]*Department of Clinical Pathology, University of Vienna, Vienna, Austria*

Abstract. *To date, no true tissue specific antigen has been discovered. Prostate-specific antigen (PSA) was initially reported to be a tissue specific protein, detected in the seminal fluid and produced by normal and abnormal epithelial cells of the prostate gland. PSA is a 33 kD glycoprotein, with serine protease activity, and it is produced by several different tissues in the human body. Its expression levels may be elevated during benign and neoplastic cell growth in the prostate, and in a number of other human malignancies. The detection of PSA is also useful in monitoring the efficacy of anticancer treatment in malignant prostatic adenocarcinoma. In the present immunocytochemical study, PSA expression was examined employing a biotin-streptavidin based, alkaline phosphatase conjugated antigen detection technique in 16 routine, neutral formalin fixed, paraffin-wax embedded, primary BC tissue sections. Human postnatal thymic tissue, among others, was used as a negative tissue control, while normal prostate and prostate carcinomas (PCs) were included in the collection of antigen positive tissues. We observed the presence of PSA in all 16 BC cases, and this expression was independent of estrogen receptor status. The intensity of the staining was moderate to high (B to A) and localized to 20% to 40% of the total BC cell population, with cells of similar immunoreactivity being clustered in groups within the tumor microenvironment. This result directly contradicts the previous opinion concerning the prostate epithelium specificity of PSA expression and production. The immunophenotype (IP) heterogeneity of BC cells is further substantiated by their PSA positivity and its association with the presence of steroid hormone receptors. The establishment of the clinical significance of these findings necessitates further in vivo and in vitro research in BCs. The prognostic significance of PSA in BCs may lie in the identification of a subset of estrogen receptor negative BC patients who have malignancies associated with a good prognosis. PSA related, novel antineoplastic immunotherapy may also be recommended.*

Correspondence to: Bela Bodey, MD, PhD, 15745 Saticoy Street, Van Nuys, CA 91406, U.S.A.

Key Words: Prostate specific antigen (PSA), PSA immunoreactivity, breast cancer (BC), Oral contraceptive induced PSA expression, steroid hormone receptors, progesterone receptors (PRs), estrogen receptors (ERs), monoclonal antibody (MoAB), immunocytochemistry, biotin-streptavidin technique, alkaline phosphatase (AP) conjugation, immunophenotype (IP); BC cell heterogeneity.

Since its first description in seminal fluid in 1971, a large amount of new information has been collected concerning the biology and expression of prostate-specific antigen (PSA). PSA (p30) provides an excellent serum marker for prostate cancer, the most frequent cancer among the American male population (1).

The mature form of PSA is a 33 kD, 237 amino acid glycoprotein, (consisting of 93% amino acids and 7% carbohydrates) thought to be produced exclusively by prostatic epithelial cells (2). PSA represents a serine protease, and it is a member of the kallikrein gene family, located on chromosome 19 (3). PSA has a high sequence homology with human glandular kallikrein 1 (hGK-1). It has chymotrypsin-, trypsin-, and esterase-like activities. In human serum, it is present in a complex form with α1-antichymotrypsin (ACT), although complex formation is slow between them *in vitro*. PSA also forms stable heteromers with α2-macroglobulin (α2M) *in vitro* but the complex does not display any of the immunogeneic PSA antigenic epitopes as a result of PSA encapsulation. The *in vivo* significance of such complex formation is not yet understood. PSA is mainly responsible for gel dissolution in freshly ejaculated semen by proteolysis of the major gel-organizing proteins semenogelin I and II and fibronectin (4).

Prostate specific antigen in normal human tissues and fluids. To date, the production of PSA has only been reported in two normal human tissues: breast epithelium and endometrium. The milk of lactating women, as well as the amniotic fluid also

contain PSA (5). Significant advances have been made regarding the identification and characterization of the molecular isoforms of PSA. In the seminal fluid, PSA seems partially connected to a serpinc (a protein C inhibitor), whereas in normal male serum it is predominantly associated with α1-antichymotrypsin (molecular weight ~100 kD) and to a smaller degree quantity with α2-macroglobulin.

In vitro studies of PSA: Several compounds such as retinoic acid and various growth factors may influence PSA protein production. It seems to be mostly under the control of circulating androgens acting through their androgen receptors. Yu and co-workers (6) were able to induce PSA production in steroid hormone receptor positive, established breast carcinoma cell lines T-47D and MCF-7 by treatment with androgens, progestins, mineralocorticosteroids, glucocorticosteroids, and antiestrogens. Estrogens failed to induce such production in both cell lines and, in addition, PSA induction was blocked by use of androgens in the T-47D cell line. The effect of PSA on estronesulphatase and estrogen 17-oxidoreductase activities were also investigated (7). PSA in low concentrations was found to inhibit the growth of the MCF-7 BC cell line and stimulated the conversion of estradiol to the less potent estrogen estrone in these cells. PSA had no effect on the MDA-MB-23 I hormone independent human BC cell line (7).

PSA in non-prostate human malignancies. During the past two years, numerous articles have been published which describe the expression of PSA in non-prostate human tumors: salivary gland neoplasms (8,9); breast cancer (6,10,11); established breast cancer cell lines (6); lung adenocarcinoma (12); parotid, liver, adrenal, kidney, colon tumors (5); ovarian carcinomas (5,13); Skene's paraurethral glands and ducts adenocarcinoma (14,15); and adenocarcinoma of the female urethra (16). Yu and coworkers (6) have reported that about 30% of BCs (from 525 cytosols observed) actually produce a 33 kD protein similar to seminal PSA. They also found PSA production at the ovarian metastatic site of the BCs and expressed the view that the detection of PSA in BCs carries with it a favorable prognosis. In the same study, ovarian cancers were also observed and it was found that from 99 primary ovarian cancers only three produced low amounts of PSA. PSA immunoreactivity was associated only with the expression of progesterone receptors (PRs) and no correlation was detected with the presence of estrogen receptors (ERs). Since 5 out of 6 male BCs were found to be negative for PSA immunoreactivity, it was suggested that androgens may not be involved in the production of PSA in BCs.

In view of the literature the specific aims of the present study were to: a) examine the usefulness of highly sensitive immunohistochemical techniques to detect the presence of PSA in human BC cells; b) determine the pattern of PSA immunoreactivity within the heterogeneous tumor cell microenvironment employing a highly specific anti-PSA MoAB (*i.e.* whether the immunoreactivity is homogeneous or localized to distinct cell clones of BC cells); and c) elaborate on the possible pathogenetic significance of PSA expression in BCs.

Materials and Methods

Tissues. In this immunocytochemical screening we used 16 BC tissues fixed in 10% neutral formalin (17) and embedded in paraffin-wax. The BC tissue sections were derived from patients treated at the University of Southern California and the breast carcinoma diagnosis was established and confirmed by staff pathologists in the Department of Pathology. Paraffin-wax BC tissue sections (3 to 4 μm thick) were cut employing a Reichert microtome and mounted on precleaned standard histological slides.

Antibodies. During this immunocytochemical observation a recently developed, anti-human mouse monoclonal antibody (MoAB) against prostate specific antigen (PSA) was employed (from Neomarkers, Fremont, CA 94, U.S.A.). Working dilution: 1:60 to 1:100. To achieve optimal immunoreactivity, tumor tissue fixation time was shortened to 4-6 hours and antigen retrieval technique was applied.

Antigen retrieval. In this immunocytochemical research project, we employed the immunohistochemical procedure of heat induced epitope retrieval (HIER) (18-20) as modified by us. Antigen retrieval required immersion of tissue sections in a Target Retrieval Solution (DAKO Corporation, Carpinteria, CA, USA) and heating in a water bath (95°C to 99°C). Unmasking of fixed antigen epitopes was also carried out by single or combined enzymatic digestion prior to the primary antigen-antibody reaction. An increase in the quantity of detectable antigenic epitopes following HIER has been described for a number of antibodies. Immunomorphological observations also reported a significant increase in the intensity of immunostaining.

Immunoalkaline phosphatase conjugated antigen detection technique. We used the following immunoalkaline phosphatase cytochemical method, modified by us for the detection of PSA in formalin fixed, paraffin-wax embedded BC tissues (21-23). The technique is a highly sensitive, indirect, four to six step immunocytochemical method, which combines the biotin-streptavidin based ABC-method (24) with enzyme-linked (alkaline phosphatase) immunohistochemistry. Briefly, following deparaffinization in three changes of Xylene substitute (Shandon-Lipshaw, Pittsburgh, PA, USA) for 20 to 30 minutes, rehydration was carried out employing descending dilutions of alcohol (100% to 50%) to TBS. An initial blocking step using 1% glacial acetic acid mixed with the working buffer for 10 minutes was necessary to eliminate the endogenous AP activity from the tissues. Use of levamisole solution is also described in our earlier observations. As we explained earlier (23), GAA inhibition was preferred because of the possible presence of levamisole-resistant AP iso-enzyme (25). The second blocking step was conducted with a purified mixture of proteins (Shandon-Lipshaw) from various species for 5-10 minutes to block cross-reactive antigenic epitopes. Excess serum was removed from the area surrounding the sections! The tissue sections were then incubated for 90-120 minutes with the particular primary antibody. Next, incubation with the secondary antibody, which was a biotinylated, whole goat anti-mouse IgG molecule (IgG molecule diluted by ICN Biomedicals, Inc., Aurora, OH, USA) was carried out for 20 minutes. Streptavidin conjugation was accomplished by incubation with AP conjugated streptavidin for 20 minutes (26-29). The binding of the biotinylated antibody to streptavidin complexes occurs with an extremely high affinity (10-19M) (BioWhitaker, Inc., Walkersville, MD, USA). Color visualization of the primary antigen-antibody (Ag-Ab) reaction was accomplished with an

alkaline phosphatase (AP) kit I (Vector Laboratories, Burlingame, CA, USA) which contains AS-TR with Tris-HCl buffer at pH 8.2 added for 28-40 minutes to allow formation of a stable red precipitate. Sections were counterstained with a diluted solution of Gill's hematoxylin (Richard-Allan, Kalamazoo, MI, USA). The tissue slides were then dehydrated in ascending concentrations of alcohol (60% to 100%) to xylene substitute (Shandon-Lipshaw), in which they were kept overnight to ensure complete morphological clearing. The stained tissue sections were mounted using a solution specially designed for use following morphological clearing in xylene substitute (Shandon-Lipshaw).

Human tissue controls in immunohistochemistry. To accurately assess the expression of PSA in BCs, we used positive and negative tumor tissues all contained on human multitumor checkerboard tissue slides: non-small cell lung, hepatocellular, colon, prostate, ovarian carcinomas and other tissues (DAKO Corp.; Lot: 5110F) (30,31). We also stained several normal, adult human brain, tonsil, thymic, thyroid, heart, lung, spleen, liver, pancreas, small intestine, large intestine, kidney, ovary, and prostate all included in one multitissue block (DAKO Corp.; Lot: 5935B). Additional controls for all employed tissues and MoABs included:

a) omission of the primary MoAB;
b) using only the enzymatic developer solution to detect the presence of endogenous alkaline phosphatase activity; and
c) use of MOPC 21 mouse myeloma IgG, (ICN Biomedicals, Inc.) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

Evaluation of the immunocytochemical results. Qualitative and quantitative evaluations of the percent of antigen positive cells and the intensity of immunostaining were conducted using a light microscope (Olympus America, Inc., Melville, NY, USA) counting 100-200 cells from each of five to eight distinct areas in nonnecrotic BCs and positive and negative control tissues. Artifacts were avoided, while, on the other band, morphologically characteristic areas were sought out. The presence of mammary carcinoma cells with very heterogeneous IPs, the endothelial elements of small blood vessels, tumor infiltrating leukocytes and macrophages (the host's immunological effector and antigen presenting cells) required careful qualitative assessment. Non-vascular elements were also examined, but only morphologically distinct neoplastically transformed cells were scored!

Quantitative evaluation. (23) (++++) over 90% of the total cell number are positive; (+++) 50% to 90% of the total cell number are positive; (++) 10% to 50% of the total cell number are positive; (+) 1% to 10% of the total cell number are positive; (±) under 1% of the total cell number are positive; (-) negative.

Qualitative evaluation. (23) (A) very intense red staining; (B) strong red staining; (C) light red staining; (D) negative staining.

Results

We observed the expression of PSA in all 16 BC cases investigated. This immunocytochemical presence was independent of the BCs' estrogen receptor status. According to our observations, the intensity of the immunoreactivity was moderate to high (B to A) and localized to 20% to 40% of the total BC cell population. The immunoreactive malignant cells were clustered in groups within the tumor cell microenvironment and formed special tumor cell clones with a differentiated IP. The IP heterogeneity of BC cells, previously investigated in detail using immunocytochemistry with extensive libraries of MoABs, is further substantiated by their PSA positivity or negativity.

Discussion

In this systematic immunocytochemical study we detected PSA immunoreactivity in 16 out of 16 observed BC cases. The PSA immunoreactivity was localized to 20% to 40% of the total BC cell population. The division of the tumor microenvironment into PSA positive and PSA negative cell groups represents another marker of cellular IP hterogeneity in BCs. The intensity of the immunoreactivity was heterogenous, ranging from moderate to strong.

Prostate specific antigen (PSA) was proclaimed as a glycoprotein produced exclusively by prostatic epithelial tissue just three years ago by Armbruster (32). However, there is a growing amount of experimental evidence that PSA may be present in many steroid hormone stimulated tissues other than that of the prostate (5). Using *reverse transcriptase-polymerase chain reaction and DNA sequencing techniques* PSA mRNA was identified in 30% of female BCs (11,33). Employing a highly sensitive *immunofluorometric assay,* PSA presence has been defined in 23 of 43 diverse tumor tissue extracts. As we mentioned above, PSA has been identified in a number of human malignancies: 30%-40% of breast carcinomas, and in a lower percentage of tumors of the skin, parotid, salivary gland, lung, colon, liver, ovary, kidney and adrenals. *High performance liquid chromatography (HPLC)* fractioning was employed to determine the molecular weight of the highly immunoreactive tumor extracts. Whereas the vast majority of tumors showed immunoreactivity eluting at both 100 kD and 33 kD, corresponding to PSA bound to α1-antichymotrypsin and free PSA, respectively, the colon and parotid tumors displayed immunoreactivity only at the 33 kD fraction.

A significant direct correlation has been described between breast carcinoma PSA immunoreactivity and progesterone (PG) positivity (34). PSA level was measured in cytosols of normal, healthy women receiving no medication and receiving a progestin containing oral contraceptive (Brevicon). Both left and right breast tissues demonstrated high levels of immunoreactive PSA after regular oral contraceptive medication (steroid hormone stimulation). The molecular weight observation defined a PSA presence identical to that of seminal PSA (35).

PSA levels in blood serum have been examined in women with and without BC, compared between women with PSA-positive and PSA-negative BCs, as well as between women with BC prior to and following surgical removal of the tumor (36,37). The authors described no difference in the serum PSA levels of normal and BC patients in women 50 years of age or older. Yu and co-workers (38) have reported that PSA immunoreactivity declines with the age of BC patients. In addition, no differences in pre- and post-surgical serum PSA levels, nor between women with PSA-positive or PSA-

negative BCs were found. HPLC defined the presence of free PSA (33 kD) in BCs. These results again suggested that female serum PSA is not associated with BC produced PSA. An important clinical study of 174 female patients defined that the relative risk for relapse was 0.34 in women with PSA positive BCs, compared to PSA negative BC patients (39). The reduced risk of relapse was independent of lymph node status, tumor size, ER and PR expression, and other prognostic parameters.

Ectopic PSA production by BC cells metastasized to the ovary has only been reported in a single article (40), further support of the view that PSA expression is a favorable prognostic parameter in BCs. Levesque and co-workers (5) conclude that in addition to normal breast epithelium, breast carcinomas, parotid, colon, liver, kidney and adrenal tumors can also produce PSA. It appears that PSA is probably a growth factor or a growth factor regulator and it may play an important role in fetal ontogenesis (41).

The human body under normal physiological conditions is in a state of homeostasis which also includes tight regulatory control over steroid and hormone levels in the body involving strict feedback mechanisms. The condition of neoplasia, by definition, is not governed by the regulatory systems of the body and thus works to throw off the balance which, under normal conditions, is continually maintained. The increase in the production of PSA following oral contraceptive treatment is a clue to the relationship between the maintencance of hormonal balance and PSA expression. Steroid hormone production and receptor expression have been detected in the medullary reticulo-epithelial (RE) cells during prenatal thymic ontogenesis in humans (42). The presence of thymosin-α1 in the seminal and follicular fluids and its participation in germ cell function have been discussed (43). It is a distinct possibility that PSA secretion is an indicator of the alteration in steroid hormone secretion and overall regulation with certain cellular immunological consequences. We suggest that the accumulation of such modifications may ultimately result in neoplastic transformation. Our immunocytochemical data suggest that PSA can no longer be regarded as a prostate specific protein. Thus, the expression of PSA by BC cells may represent a positive prognostic marker.

Acknowledgements

The authors appreciate the assistance of Dr. Victoria Psenko in the preparation of this manuscript.

References

1 Villoutreix BO, Getzoff ED, Griffin JH: A structural model for the prostate disease marker, human prostate-specific antigen. Protein Science *3:* 2033-2044, 1994.

2 Seregni E, Botti C, Ballabio G, Bombardieri E: Biochemical characteristics and recent biological knowledge on prostate-specific antigen. Tumori *82:* 72-77, 1996.

3 el-Shirbiny AM: Prostatic specific antigen. Advances Clin Chem *31:* 99-133, 1994.

4 Lilja H: Structure, function, and regulation of the enzyme activity of prostate-specific antigen. World J Urol *11:* 188-191, 1993.

5 Levesque M, Hu H, D'Costa M, Diamandis EP: Prostate-specific antigen expression by various tumors. J Clin Lab Anal *9:* 123-128, 1995.

6 Yu H, Diamandis EP, Zarghami N, Grass L: Induction of prostate specific antigen production by steroids and tamoxifen in breast cancer cell lines. Breast Cancer Res Treat *32:* 291-300, 1994.

7 Lai LC, Erbas H, Lennard TW, Peaston RT: Prostate-specific antigen in breast cyst fluid: possible role of prostate-specific antigen in hormone-dependent breast cancer. Int J Cancer *66:* 743-746, 1996.

8 van Krieken JH: Prostate marker immunoreactivity in salivary gland neoplasms. A rare pitfall in immunohistochemistry. Am J Surg Pathol *17:* 410-414, 1993.

9 Poller D: Prostate marker immunoreactivity in salivary gland neoplasms. Am J Surg Pathol *18:* 214-215, 1994.

10 Diamandis EP, Yu H, Sutherland DJ: Detection of prostate-specific antigen immunoreactivity in breast tumors. Breast Cancer Res Treat *32:* 301-310, 1994.

11 Monne M, Croce CM, Yu H, Diamandis EP: Molecular characterization of prostate-specific antigen messenger RNA expressed in breast tumors. Cancer Res *54:* 6344-6347, 1994.

12 Bilgrami S, Singh NT, Shafi N, Ciesielski T: Raised prostate-specific antigen in adenocarcinoma of lung. Lancet *344:* 1371-1372, 1994.

13 Yu H, Diamandis EP, Levesque M, Asa SL, Monne M, Croce CM: Expression of the prostate-specific antigen gene by a primary ovarian carcinoma. Cancer Res *55:* 1603-1606, 1995.

14 Zaviacic M, Sidlo J, Borovsky M: Prostate specific antigen and prostate specific acid phosphatase in adenocarcinoma of Skene's paraurethral glands and ducts. Virchows Arch A Pathol Anat Histopathol *423:* 503-505, 1993.

15 Dodson MK, Cliby WA, Keeney GL, Peterson MF, Podratz KC: Skene's gland adenocarcinoma with increased serum level of prostate-specific antigen. Gynecol Oncol *55:* 304-307, 1994.

16 Ebisuno S, Miyai M, Nagareda T: Clear cell adenocarcinoma of the female urethra showing positive staining with antibodies to prostate-specific antigen and prostatic acid phosphatase. Urology *45:* 682-685, 1995.

17 Farmilo AJ, Stead RH: Fixation in immunocytochemistry. In: Handbook Immunochemical Staining Methods, Ed. Naish SJ, DAKO Corporation, 1989, pp. 2429.

18 Shi S-R, Key ME, Kalra KL: Antigen retrieval in formalin fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem *39:* 741-748, 1991.

19 Shi S-R, Cote C, Kalra KL, Taylor CR, Tandon AK: A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. J Histochem Cytochem *40:* 787-792, 1992.

20 Beckstead JH: Improved antigen retrieval in formalin-fixed, paraffin-embedded tissues. Appl Immunohistochem *2:* 274-281, 1994.

21 Janckila AJ, Yam LT, Li C-Y: Immunoalkaline phosphatase cytochemistry. Amer J Clin Pathol *84:* 476-480, 1985.

22 Cattoretti G, Berti E, Schiro R, D' Amato L, Valeggio C, Rilke F: Improved avidin-biotin-peroxidase complex (ABC) staining. Histol J *20:* 75-80, 1988.

23 Bodey B, Zeltzer PM, Saldivar V, Kemshead J: Immunophenotyping of childhood astrocytomas with a library of monoclonal antibodies. Int J Cancer *45:* 1079-1087, 1990.

24 Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison of ABC and unlabeled antibody (PAP) procedure. J Histochem Cytochem *29:* 577-581, 1981.

25 Yam LT, Janckila AJ, Epremian BE, Li C-Y: Diagnostic significance of levamisole-resistant alkaline phosphatase in cytochemistry and immunocytochemistry. Amer J Clin Pathol *91:* 31-36, 1989.

26 Strasburger CJ, Amir-Zaltsman Y, Kohen F: The avidin-biotin reaction as an universal amplification system in immunoassays. Prog Clin Biol Res *285:* 79-100, 1988.

27 Wilchek M, Bayer EA: Introduction to avidin-biotin technology. Methods Enzymol *184:* 5-13, 1990.

28 Duhamel RC, Whitehead JS: Prevention of nonspecific binding of avidin. Methods Enzymol *184:* 201-207, 1990.

29 Diamandis EP, Christopoulos TK: The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem *37:* 625-636, 1991.

30 Battifora H: The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest *55:* 244-248, 1986.

31 Battifora H, Mehta P: The checkerboard tissue block. An improved multitissue control block. Lab Invest *63:* 722-724, 1990.

32 Armbruster DA: Prostate-specific antigen: biochemistry, analytical methods, and clinical application. Clin Chem *39:* 181-195, 1993.

33 Zarghami N, Diamandis EP: Detection of prostate-specific antigen mRNA and protein in breast tumors. Clin Chem *42:* 361-366, 1996.

34 Ferguson RA, Yu H, Kalyvas M, Zammit S, Diamandis EP: Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers. Clin Chem *42:* 675-684, 1996.

35 Yu H, Diamandis EP, Monne M, Croce CM: Oral contraceptive-induced expression of prostate-specific antigen in the female breast. J Biol Chem *270:* 6615-6618, 1995.

36 Yu H, Giai M, Diamandis EP, Katsaros D, Sutherland DJ, Levesque MA, Roagna R, Ponzone R, Sismondi P: Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer. Cancer Res *55:* 2104-2110, 1995.

37 Giai M, Yu H, Roagna R, Ponzone R, Katsaros D, Levesque MA, Diamandis EP: Prostate-specific antigen in serum of women with breast cancer. Brit J Cancer *72:* 728-731, 1995.

38 Yu H, Diamandis EP, Sutherland DJ: Immunoreactive prostate-specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. Clin Biochem *27:* 75-79, 1994.

39 Yu H, Diamantis ER: Prostate-specific antigen (PSA) as a new prognostic marker in female breast cancer: molecular characterization and clinical implication. (Meeting Abstract) Proc Annu Meet Am Assoc Cancer Res *36:* A1464, 1995.

40 Yu H, Diamantis ER, Levesque M, Sismondi P, Zola P, Katsaros D: Ectopic production of prostate specific antigen by a breast tumor metastatic to the ovary. J Clin Lab Analysis *8:* 251-253, 1994.

41 Diamantis ER: New diagnostic applications and physiological functions of prostate specific antigen. Scand J Clin Lab Invest (Suppl) *221:* 105-112, 1995.

42 Bodey B: Histomorphology and histochemistry of the human thymus during its prenatal ontogenesis. Dissertation, Inst Morphol, Bulg Acad Sci, Sofia, Bulgaria, 1977, pp 1-360.

43 Naz RK, Naylor PH, Goldstein AL: Thymosin α1 levels in human seminal plasma and follicular fluid: implication in germ cell function. Int J Fertil *32:* 375-379, 1987.

Received November 11, 1996
Accepted February 3, 1997

Multiple Marker Evaluation in Human Prostate Cancer With the Use of Tissue-Specific Antigens [1,2,3]

M. Kuriyama, [4] M. C. Wang, [4] C. L. Lee, [4] C. S. Killian, [4] L. D. Papsidero, [4] H. Inaji, [4] R. M. Loor, [4] M. F. Lin, [4] T. Nishiura, [5] N. H. Slack, [6] G. P. Murphy, [6] *and* T. M. Chu [4,7,8]

ABSTRACT—Serum prostate-specific antigen and prostatic acid phosphatase were simultaneously evaluated in 22 healthy males, 29 patients with benign prostatic hypertrophy, and 192 patients with prostate cancers at various stages as well as in 30 patients with cancers other than prostate cancer. Both markers were quantitated by specific sandwich-type, enzyme-linked, immunosorbent assays with the use of specific antiserum reagents. Serum assays revealed a discordance between these two markers; thus expressions of these two biochemically and immunologically distinct prostate-specific proteins may reflect different aspects in the biology of prostate cancer. A combination test with the use of 7.5 ng of prostate antigen and 15.5 ng of prostatic acid phosphatase/ml of serum, respectively, as cutoff values resulted in a positive detection rate of 58% for prostate cancers of stages A (7/12) and B (21/36) each, 68% for prostate cancer of stage C (19/28), 92% for prostate cancer of stage D (106/116), and only 10% for benign prostatic hypertrophy (3/29). None of 52 other cancers or healthy controls was registered as positive. This study demonstrates that a multiple marker test of tissue-specific antigens can be of an additive value in the immunodiagnosis of cancer and may be a logical and effective approach at this time, in light of the unavailability of human tumor-specific markers.—JNCI 1982; 68:99-105.

Assay of acid phosphatase activity (EC 3.1.3.2) has been used as an aid in the diagnosis of prostate cancer, since Gutman et al. (*1*) in 1936 reported an association between elevated serum acid phosphatase and metastatic prostate cancer. Recently, several immunochemical procedures have been developed for quantitation of serum PAP (*2-8*). By these immunoassays with antisera specific to PAP, various results with regard to the detection of elevated serum PAP in prostate cancer have been reported.

This laboratory reported 2 years ago the identification and purification of a new antigen, PA (*9*). By an immunocytochemical technique, PA has been shown to be localized in the cytoplasm of prostatic ductal cells (*10*). Human prostate tumor cells in vitro and in vivo also express this antigen (*11*). Circulating PA in patients with prostate cancer has been shown to be biochemically similar and immunologically identical to that of prostate tissue (*12*). Further, a sensitive sandwich-type ELISA has been developed for quantitation of PA (*13*). PA was not detectable in sera from healthy females or from female cancer patients. Sera from male patients with cancers other than prostate cancer contained a range of PA similar to that of healthy males. However, patients with prostate cancer had significantly elevated levels of serum PA. Since PA is biochemically and immunologically distinct from PAP (*9, 11*), the expressions of these two prostate-specific proteins may be unique parameters in combination tests for serologic detection of prostate cancer. The data may provide useful information for the design of multiple marker studies for cancer. Also, new potential uses of tissue- or organ-specific antigens, rather than tumor-specific antigens, may be realized in the immunodiagnosis of cancer. This report describes our initial evaluation on the simultaneous measurements by two ELISA procedures of serum PA and PAP in prostate cancer.

ABBREVIATIONS USED: BPH = benign prostatic hypertrophy; BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; PA = human prostate-specific antigen; PAP = human prostatic acid phosphatase; PBS = phosphate-buffered saline [0.01 *M* NaH_2PO_4–0.01 *M* Na_2HPO_4–0.154 *M* NaCl (pH 7.1)]; pI = isoelectric point(s).

---

[1] Received April 24, 1981; accepted August 20, 1981.

[2] Supported in part by Public Health Service grants CA-15437, CA-15126, and CA-23990 from the National Cancer Institute.

[3] Research procedures were in accord with the ethical standards of the Human Experimentation Committee, Roswell Park Memorial Institute.

[4] Department of Diagnostic Immunology Research and Biochemistry, Roswell Park Memorial Institute, 666 Elm St., Buffalo, N.Y. 14263.

[5] Department of Urology, Gifu University School of Medicine, Gifu, Japan.

[6] National Prostatic Cancer Project, Roswell Park Memorial Institute. Other investigators include: Dr. D. Johnson, M. D. Anderson Hospital and Tumor Institute, Houston, Tex.; Dr. S. A. Leoning, University of Iowa Hospitals and Clinics, Iowa City, Iowa; Dr. R. P. Gibbons, The Mason Clinic, Seattle, Wash.; Dr. G. R. Prout, Massachusetts General Hospital, Boston, Mass.; Dr. W. W. Scott, The Johns Hopkins University Hospital, Baltimore, Md.; Dr. M. Soloway, University of Tennessee, Memphis, Tenn.; Dr. J. D. Schmidt, University of California at San Diego, San Diego, Calif.; Dr. S. Bergman, Tulane Medical Center, New Orleans, La.; Dr. J. M. Pierce, Hutzel Hospital/Wayne State University, Detroit, Mich.; Dr. P. Scardino, Baylor University, Houston, Tex.; Dr. M. McLeod, Walter Reed Army Medical Center, Washington, D.C.; Dr. C. McKiel, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Ill.; Dr. J deKernion, University of California at Los Angeles, Los Angeles, Calif.; Dr. S. Beckley, Roswell Park Memorial Institute; and Dr. E. J. Pontes, Roswell Park Memorial Institute, formerly at Hutzel Hospital/Wayne State University.

[7] *Address reprint requests to* Dr. Chu.

[8] We acknowledge the valuable secretarial assistance of Ms. J. Ogledzinski and Ms. S. Witalis.

MATERIALS AND METHODS

Materials.—HRP (EC 1.11.1.7; type VI, RZ3.2, lot #59C-9760), 2,4-dinitrofluorobenzene, sodium borohydride, *o*-dianisidine, poly-L-lysine succinate, and BSA were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium metaperiodate and monoethanolamine were obtained from Fisher Scientific Co., Fair Lawn, N.J. Sephadex G-200 was the product of Pharmacia Fine Chemicals, Piscataway, N.J.

Normal human sera were collected from apparently healthy volunteers 24-65 years old. Serum specimens from patients with histologically confirmed prostate cancers and BPH were received from the National Prostatic Cancer Project's Serum Bank and Gifu University School of Medicine, respectively. Prostate cancers were staged by investigators of the National Prostatic Cancer Project (*14*). All patients had active disease, although some patients were receiving treatment. Most patients with stages A, B, and C cancer had had no treatment. All patients with advanced stage D were refractory to hormone or radiation therapy and had not yet been given chemotherapy. Sera from patients with other cancers were obtained from Roswell Park Memorial Institute; all of these patients had metastatic cancer. Specimens were stored at −85°C until used.

Immunologic reagents.— Preparation of purified PA and PAP and production of specific anti-PA and anti-PAP antisera in the goat were reported previously (*3, 6, 9, 12*). The IgG fraction of goat immune sera was prepared by ammonium sulfate precipitation and Sephadex G-200 gel filtration (*13*). The IgG fraction from goat normal serum was isolated in the same manner. Coupling of HRP to anti-PA IgG and anti-PAP IgG was described previously (*13*).

Coating of anti-PAP to tube.—Coating of anti-PAP IgG to a polystyrene tube (12×75 mm) was done by the procedure of Kelsoe and Weller (*15*). Each tube was incubated first with 200 μl of solution containing 0.25 mg of poly-L-lysine succinate/ml for 15 minutes at room temperature. The tube was washed three times with 1 ml of PBS, after which 200 μl of anti-PAP IgG or normal goat IgG in 0.01 *M* carbonate buffer (pH 9.6; 50 μg/ml or 25 μg/ml) was added to each tube and each tube was incubated at 37°C for 24 hours. Then 1 ml of 1% BSA in PBS was added to the tube to saturate any uncoated sites. After 3 hours of incubation at 37°C, the antibody-coated tube was stored at 4°C until used.

Quantitation of PAP.—Purified PAP or a diluted serum sample (200 μl) was incubated in an anti-PAP-coated tube for 3 hours at 37°C. To each tube was added 1 ml of washing buffer (PBS containing 1% BSA and 0.01 *M* EDTA:Na salt). After gently whirling in a vortex, 1 ml of the solution was removed. This washing step was repeated three times. HRP-labeled anti-PAP IgG (100 μl) was then added, and the tube was incubated for another 3 hours at 37°C, which resulted in the formation of anti-PAP IgG:PAP:anti-PAP IgG-HRP. After the tube was washed three times, the peroxidase activity of the complex was then quantitated as described previously at 403 nm (A_{403}) (*13*). The percentage bound was calculated from $[(B-Bo)/(B_t-Bo)]\times 100$, where $B=A_{403}$ of the sample, $B_t=A_{403}$ of maximum, and $Bo=A_{403}$ of the blank. Each assay was performed in duplicate with two controls, one blank (without sample) and the other containing purified PAP at a concentration of 10 μg/ml for the maximal binding. The quantity of PAP in the specimen was determined from a standard curve constructed in an identical manner with the use of various concentrations of purified PAP.

The reproducibility of this procedure was evaluated by the determination of the coefficients of variation between assays (i.e., two samples containing different concentrations of PAP were assayed in 10 separate experiments) and by the determination of the variation within assays (i.e., two other samples with different PAP levels were analyzed in a series of 10 assays each) (*13*).

Nonspecific adsorption of the tube-coated anti-PAP IgG.—A total of 27 normal male sera were tested for nonspecific adsorption to the insolubilized antibody with the use of goat normal IgG coated to the tube.

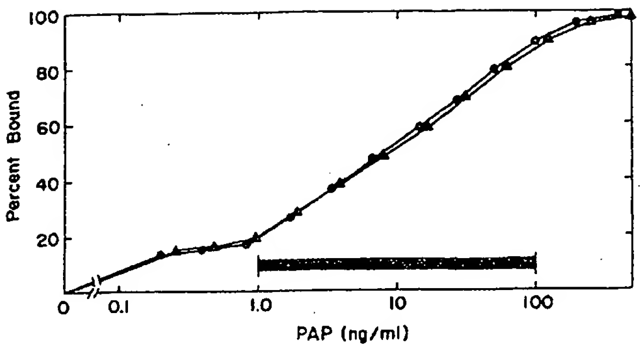
Quantitation of PA.—Serum PA levels were determined by the ELISA procedure as reported recently (*13*); by this procedure, anti-PA IgG was conjugated to a solid-phase support, CNBr-activated Sepharose 4B. After incubation of the specimen with Sepharose 4B-anti-PA, the resulting Sepharose-anti-PA:PA was further reacted with anti-PA-HRP. The HRP in the sandwich-type Sepharose 4B-anti-PA:PA:anti-PA-HRP was then quantitated and used as a parameter for serum PA concentration.

RESULTS

ELISA of PAP

The newly developed, tube-supported, sandwich-type ELISA of PAP, as performed by the experimental protocol described, yielded a working range of 1.0-100 ng of PAP/ml of serum sample (text-fig. 1). Quality-control data of within-assay variation (two levels of PAP, 6.5 and 35, and 10 assays each) and between-assay variation (two levels of PAP, 2.6 and 51, and 10 assays each) revealed an overall mean coefficient of variation of 4.3% (ranging from 3.2 to 5.7%), indicating that this new enzyme immunoassay for PAP was a reproducible procedure. With the use of a tube coated with goat preimmune IgG, rather than with goat immune anti-PAP IgG, 27 serum specimens of healthy males were shown to have levels of nonspecific binding from 15 to 19% (mean, 17.4%). The ranges represented approximate values of 1.0 ng or less, the sensitivity limit of this ELISA (text-fig. 1). Therefore, the PAP assay was performed without the simultaneous use of a control, e.g., a preimmune goat IgG-coated tube.

In addition, sera from a group of 27 apparently



TEXT-FIGURE 1.—Standard calibration curve of PAP as determined by the ELISA with the use of a hundredfold-diluted HRP-anti-PAP IgG and 10 μg of anti-PAP IgG applied to a polystyrene tube. ●, purified PAP; ▲, serum containing 480 ng PAP/ml as standard. *Horizontal bar* indicates the working range of this ELISA. Percent bound was calculated by the formula $[(B-Bo)/(B_t-Bo)]\times 100$, where $B=A_{403}$ of the sample, $B_t=A_{403}$ of the maximum, and $Bo=A_{403}$ of the blank as indicated in the text.

healthy normal male controls were shown by this ELISA to have PAP levels ranging from less than 1.0 to 13 ng/ml with a mean plus or minus standard deviation of 4.4±3.7. For statistical analysis, an upper cutoff point for the normal range was, therefore, calculated as 15.5 ng PAP/ml of serum (mean + 3 SD).

Single Assay of PAP

Serum PAP values in patients with prostate cancers, BPH, and other cancers are shown in table 1. Thirty male patients with cancers of the lung, pancreas, and colon-rectum (10 each) showed a range of serum PAP similar to that seen in 27 healthy males, as did 29 patients with BPH. A significantly higher mean serum PAP value, however, was obtained from patients with all clinical stages of prostate cancers. The mean of serum PAP in 12 patients with stage A prostate cancer, 15.5 ng/ml, was equal to the upper cutoff point for the range in 27 healthy males. In addition, the mean serum PAP increased with increasing disease stage. Serum PAP levels were elevated in 33% of stage A, 42% of stage B, 64% of stage C, and 73% of stage D prostate cancer patients. Text-figure 2 shows a scattergram of the data presented in table 1 for males.

This study also included serum "PAP" values from 10 healthy female controls and 19 female cancer patients. As table 1 shows, all mean values were less than 1.5, representing circulating acid phosphatase of non-prostate origin or other proteins that may react nonspecifically with goat anti-PAP serum by the ELISA procedure, although anti-PAP serum used in the assay previously has been shown by immunoprecipitation and immunocytochemical techniques to be specific to acid phosphatase of prostate origin.

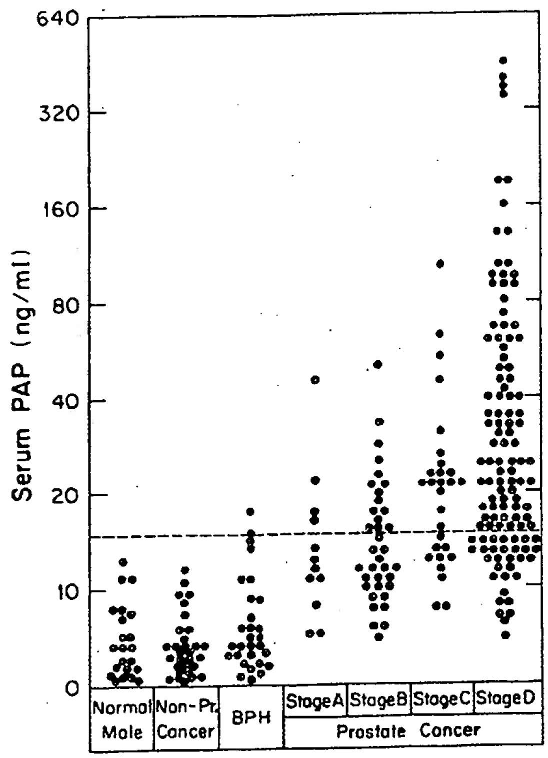
Single Assay of PA

The PA assay also was performed in serum specimens at the time that PAP levels were determined. For statistical analysis, an upper cutoff value also was calculated from the means + 3 SD of healthy controls, which yielded a value of 2.5 ng PA/ml of serum. As reported previously, the sensitivity of our ELISA of PA was 0.10 ng/ml, and sera of all females, either healthy controls or cancer patients, contained PA less than 0.10 ng/ml (as further demonstrated in the 29 specimens assayed in this study). Again, with one exception, male patients with cancers other than prostate cancer had serum PA values of less than 2.5 ng/ml. Although 41% (12/29) of the BPH patients exhibited elevated serum PA levels, more prostate cancer patients [67% (8/12) with stage A cancer, 69% (25/36) with stage B, 79% (22/28) with stage C, and 83% (96/116) with stage D] demonstrated an elevated PA with a higher mean value

TABLE 1.—*Serum PAP level by ELISA in various diseases*

Groups	No. of persons examined	Serum PAP, ng/ml: Male, No. of males	Male, Range	Male, Mean ± SD	Male, >15.5 (%)	Female, No. of females	Female, Range	Female, Mean ± SD
Healthy subjects	37	27	<1.0–13	4.4±3.7	0	10	<1.0–2.4	1.3±0.6
Patients with cancers other than prostate cancer[a]	49	30	<1.0–12	4.2±3.2	0	19	<1.0–3.6	1.4±0.7
Lung cancer	15	10	1.0–12	4.4±3.3	0	5	<1.0–2.3	1.5±0.6
Breast cancer	5					5	<1.0–2.2	1.3±0.5
Pancreatic cancer	14	10	1.0–11	4.0±2.8	0	4	<1.0–3.6	1.5±1.2
Colorectal cancer	15	10	<1.0–9.9	4.3±3.4	0	5	<1.0–2.2	1.4±0.5
Patients with BPH	29	29	<1.0–18	6.0±4.8	2 (7)			
Patients with prostate cancers of[b]								
Stage A	12	12	5.3–48	15.5±10.9	4 (33)			
Stage B	36	36	5.0–55	15.9±9.4	15 (42)			
Stage C	28	28	8.4–110	26.0±21.4	18 (64)			
Stage D	116	116	4.7–480	65.3±86.1	85 (73)			

[a] All with metastatic cancer.
[b] All with disease present. Most patients with stages A, B, and C cancers had not yet been treated. All patients with stage D cancer were refractory to hormone or radiation therapy and had not yet received chemotherapy.



TEXT-FIGURE 2.—Serum PAP levels in healthy male controls (Normal Male), male patients with cancers other than prostate cancer (Non-pr. Cancer), patients with BPH, and patients with prostate cancers of various stages. *Broken horizontal line* shows a PAP level of 15.5 ng/ml, the upper limit of the normal range (mean + 3 SD).

(text-fig. 3). In comparison to the PAP assay, the PA assay registered positive values more often in the BPH patients.

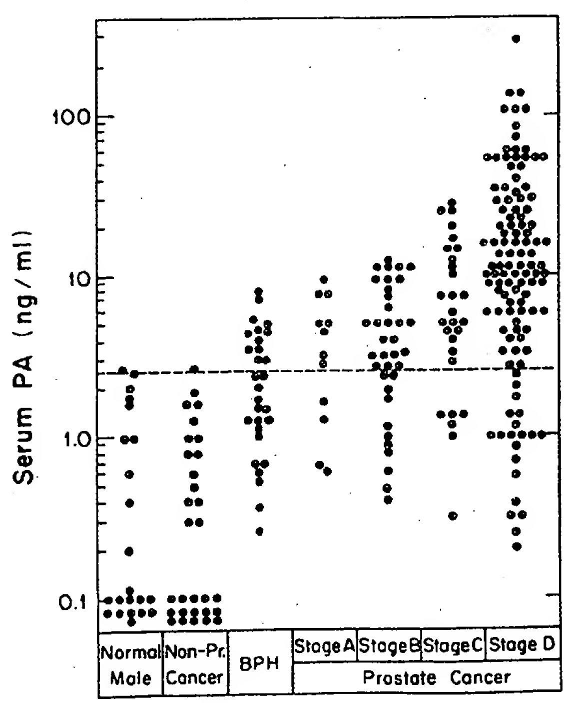
Multiple Assays of PA and PAP

Among 22 healthy male controls, 29 BPH patients, 192 prostate cancer patients, and 30 males with other cancers, assays of both PA and PAP were performed simultaneously. The elevation of either PA or PAP levels was then determined. Overall, 64 and 79% of 192 patients with prostate cancers had elevated PAP and PA levels, respectively. As already noted, an elevated PA also was shown in a higher percentage of the patient population. It was found in 67% of the patients with stage A prostate cancer, 69% with stage B, 79% with stage C, 83% with stage D, and in 41% of the patients with BPH. In comparison with the elevations in PA levels, the levels of PAP were elevated in 34, 41, 64, and 74% of the patients with stages A, B, C, and D prostate cancers, respectively, and in 7% of the patients with BPH. For both assays, 83% of the patients with stage A cancer to 94% of the patients with stage D were shown to have elevated PA and/or PAP values, although 48% of the patients with BPH also registered as positive in either PA or PAP assay.

In practice, for multiple marker study using PA and PAP, one may increase the upper cutoff point of PA ~~to~~ a value of the mean + 2 SD, i.e., 7.5 ng/ml, calculated from BPH patients. In this manner, the sensitivity of the PA assay was decreased, but the specificity was increased inasmuch as the false positives presented by BPH were almost eliminated. With this adjustment, table 2 shows a very promising result for simultaneous determinations of PA and PAP in prostate cancer. When both assays were combined, only 10% of the BPH patients were positive in the test. Also, 153 (79%) of 192 prostate cancer patients were positive for either PA or PAP, ranging from 58% for patients with stages A and B each, to 68% for patients with stage C, to 92% for patients with stage D; a significant number (53) of the patients were positive with both assays. Furthermore, patients with other cancers or healthy controls registered no positive assay. Therefore, these results as derived from two prostate tissue antigens that were measured simultaneously clearly demonstrated an additive value.

DISCUSSION

Data obtained from simultaneous assays of two prostate-specific markers, PA and PAP, have been presented. Serum PA is quantitated by an established solid-phase sandwich-type (Sepharose 4B-anti-PA:PA: anti-PA-HRP) ELISA (*13*), whereas PAP is measured



TEXT-FIGURE 3.—Serum PA levels in healthy male controls, male patients with cancers other than prostate cancer (Non-Pr. Cancer), patients with BPH, and patients with prostatic cancers of various stages. *Broken horizontal line* indicates a PA level of 2.5 ng/ml, the upper limit of the normal range (mean + 3 SD).

TABLE 2.—*Simultaneous assay of serum PA and PAP*

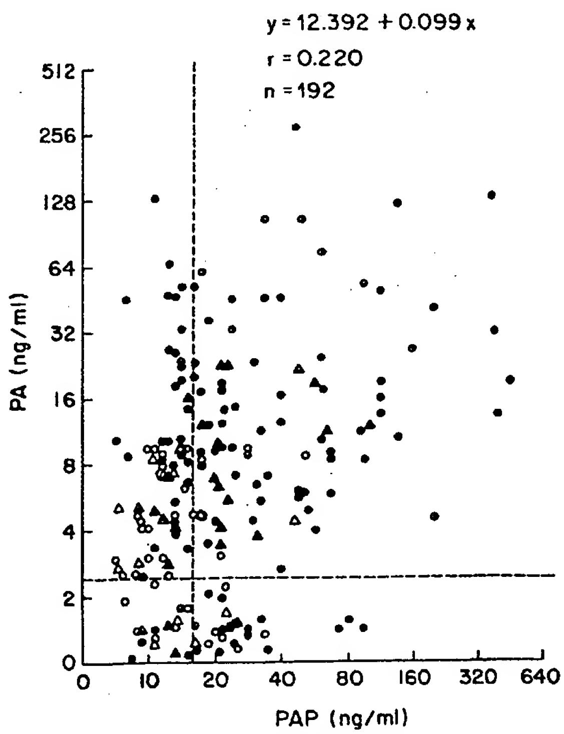
Groups	No. of persons examined	Assay results[a]			
		PAP (−) and PA (−)	PAP (−) and PA (+)	PAP (+) and PA (−)	PAP (+) and PA (+)
Healthy males	22	22 (100)	0 (0)	0 (0)	0 (0)
Patients with cancers other than prostate cancers[b]	30	30 (100)	0 (0)	0 (0)	0 (0)
BPH patients	29	26 (90)	1 (3)	2 (7)	0 (0)
Patients with prostate cancers of[c]	192	39 (20)	31 (16)	56 (29)	66 (34)
Stage A	12	5 (42)	3 (25)	4 (33)	0 (0)
Stage B	36	15 (42)	6 (17)	11 (31)	4 (11)
Stage C	28	9 (32)	1 (4)	9 (32)	9 (32)
Stage D	116	10 (8)	21 (18)	32 (28)	53 (46)

[a] Results are expressed as No. of individuals in each category (%). PAP, <15.5 ng/ml (−) or >15.5 ng/ml (+); 15.5 = mean + 3 SD of normal values. PA, <7.5 ng/ml (−) or >7.5 ng/ml (+); 7.5 = mean + 2 SD of BPH values.
[b] All with metastatic cancer.
[c] All with disease present. Most patients with stages A, B, and C cancers had not yet been treated. All patients with stage D cancer were refractory to hormone or radiation therapy and had not yet received chemotherapy.

by a newly reported, tube-supported, sandwich-type (tube-anti-PAP:PAP:anti-PAP-HRP) ELISA. The ELISA of PAP detects an antigenic protein of the enzyme, unlike our several other immunochemical procedures, such as counterimmunoelectrophoresis (*3, 8*), fluorescent immunoassay (*4*), immunoadsorbent assay (*6*), and the double-antibody immunoenzyme assay of Choe et al. (*5*), which detect both antigenic sites and the hydrolytic activity of the enzyme. In this ELISA of PAP, the HRP activity of the final conjugate, tube-anti-PAP:PAP:anti-PAP-HRP, is measured and serves as an index for quantitation of PAP. This assay detects more elevated serum PAP in the early stage of prostate cancer than does the counterimmunoelectrophoresis technique (*3, 8*) but reveals a similar detection rate as the fluorescent immunoassay (*4*) or the immunoadsorbent assay (*6*).

Both PA and PAP are proteins of human prostate-specific origin (*3, 9*). Biochemically, PA is a glycoprotein of molecular weight 33,000 with a pI of 6.9, whereas PAP is a larger, 100,000, glycoprotein with multiple pI ranging from 4.2 to 5.5 (*3, 9, 16*). Immunologically, PA and PAP are two distinct antigen moieties; i.e., antisera possess specific reactivity against their respective antigens and demonstrate no cross-reactivity with each other (*12*). PA is not a subunit or a related peptide of PAP, because both proteins exhibit different amino acid compositions and peptide mapping (*17*). As text-figure 4 shows, different levels of PA and PAP are expressed by patients with prostate cancer; i.e., in some patients PAP levels are in the normal range, while PA levels are elevated. Further, although not prostate tumor-specific, PA and PAP have been shown to be promising markers in the detection of prostate cancer; they may reflect different aspects of neoplastic transformation in prostate cancer. These observations are the rationale for this study.

In evaluating the possible role of serum PA and PAP assays in the immunodiagnosis of prostate cancer, we have used the conventional mean + 3 SD of male controls as the upper normal limits for serum PA and PAP values. Under these criteria, each assay alone detected an elevation of PAP and PA in 64 and 79% of prostate cancer patients, respectively. None of the healthy males and 7% of the BPH patients had an elevated PAP, whereas 9% of the healthy males, 3% of the patients with cancers other than prostate cancer, and 41% of the BPH patients demonstrated an elevated PA value. In the combination test, these individuals also registered positive assay values. However, in pros-



TEXT-FIGURE 4.—Simultaneous assays of serum PA and PAP in 192 randomly selected prostate cancer patients. *Two broken lines* indicate the upper normal limits of serum PA and PAP. Δ, stage A; O, stage B; ▲, stage C; ●, stage D. y, PA; x, PAP. r = correlation coefficient; n = number.

tate cancer, a combination of these two assays increased the positive detection rate of PA and/or PAP to 83% for the patients with stage A prostate cancer, 89% for the patients with stages B and C, and 94% for the patients with stage D. The PA assay alone and the PAP assay alone gave respective rates of 57, 69, 79, and 80% and 33, 42, 64, and 73% for patients with stages A, B, C, and D prostate cancers. An elevated PA level still was detected in 41% of the BPH patients as indicated.

As already indicated, one advantage of a combination test with the use of prostate-specific markers is the increase in the sensitivity of detection of an elevation of either marker in prostate cancer. It is of concern that 41% of the BPH patients exhibited an elevated PA. Another obvious advantage in the use of a combination test is that adjustment can be made of various cutoff points for each marker assay and comparisons can be done of the sensitivity and specificity of the combined test. In this study, the cutoff of PA is calculated as the level of the mean + 2 SD for BPH, 7.5 ng/ml, and used for analysis of combined PA and PAP assays. Comparison of the sensitivity and specificity of the combination test results in an impressive figure, as shown in table 2; 58% each of the patients with stages A and B prostate cancers, 68% of the patients with stage C, and 92% of the patients with stage D prostate cancers (overall, 80%) and only 10% of the patients with BPH demonstrated positive PA and/or PAP levels. In addition, no healthy control or patients with cancers other than prostate cancer exhibited positive levels of PA and/or PAP under this set of parameters, i.e., when cutoff points of means + 3 SD in PAP from healthy males and means + 2 SD in PA from BPH patients are combined.

At present, the possible use of the serum PAP assay, specifically by the radioimmunoassay technique, has generated much debate, entailing such considerations as specificity, sensitivity, and predictive value for screening of prostate cancer (*18-20*). We cannot address or speculate on these complex and critical questions with data available from this report. However, our results clearly show that the measurements of PAP and PA together have an additive effect on the diagnosis of prostate cancer. What usefulness this fact will be clinically requires more data, particularly those derived from patients with early prostate cancer whose cancers are staged surgically. Probably, the most important clinical application of the PAP and/or PA immunochemical assay at its present stage of development is for the monitoring of prostate cancer (*21, 22*).

In conclusion, a multiple marker assay in prostate cancer has been evaluated with the use of PA and PAP. PA and PAP are two biochemically and immunologically distinct proteins of human prostate tissue. Although not tumor-specific, PA and PAP are tissue-specific markers and therefore were used in our study. Results from serum assay revealed a discordance between the PA and PAP values, which thus suggested that the expressions of these two markers reflect different aspects of the biology of prostate cancer. Combination tests with different and selected values of PA and PAP levels produced very encouraging results. This study demonstrates that a combination test of tissue-specific markers can result in additive clinical value in the immunodiagnosis of cancer and should be a logical avenue for serious consideration at this time in light of the absence of human tumor-specific markers.

REFERENCES

(*1*) GUTMAN EB, SPROUL EB, GUTMAN AB. Significance of increased phosphatase activity of bone at site of osteoplastic metastasis secondary to carcinoma of prostate gland. Am J Cancer 1936; 28:485-495.

(*2*) FOTI AG, HERSCHMAN H, COOPER JF. A solid-phase radioimmunoassay for human prostatic acid phosphatase. Cancer Res 1975; 35:2446-2452.

(*3*) CHU TM, WANG MC, SCOTT WW, et al. Immunochemical detection of serum prostatic acid phosphatase. Methodology and clinical evaluation. Invest Urol 1978; 15:319-322.

(*4*) LEE CL, WANG MC, MURPHY GP, CHU TM. A solid-phase fluorescent immunoassay for human prostatic acid phosphatase. Cancer Res 1978; 38:2871-2878.

(*5*) CHOE BK, PONTES JE, DONG MK, ROSE NR. Double antibody immunoenzyme assay for human prostatic acid phosphatase. Clin Chem 1980; 26:1854-1858.

(*6*) LEE CL, KILLIAN CS, MURPHY GP, CHU TM. A solid-phase immunoadsorbent assay for serum prostatic acid phosphatase. Clin Chim Acta 1980; 101:209-216.

(*7*) VIHKO P, SAGANTI E, JANNE O, PELTONEN L, VIHKO R. Serum prostate-specific acid phosphatase: Development and validation of a specific radioimmunoassay. Clin Chem 1978; 24: 1915-1919.

(*8*) KILLIAN CS, VARGAS FP, LEE CL, et al. Quantitative counterimmunoelectrophoresis assay for prostatic acid phosphatase. Invest Urol 1980; 18:219-224.

(*9*) WANG MC, VALENZUELA LA, MURPHY GP, CHU TM. Purification of a human prostate specific antigen. Invest Urol 1979; 17:159-163.

(*10*) NADJI M, TABEI SZ, CASTRO A, et al. Prostatic specific antigen: An immunohistologic marker for prostatic neoplasm. Cancer 1981; 48:1229-1232.

(*11*) PAPSIDERO LD, KURIYAMA M, WANG MC, et al. Prostate antigen: A marker for human prostate epithelial cells. JNCI 1981; 66:37-42.

(*12*) PAPSIDERO LD, WANG MC, VALENZUELA LA, MURPHY GP, CHU TM. A prostate antigen in sera of prostatic cancer patients. Cancer Res 1980; 40:2428-2432.

(*13*) KURIYAMA M, WANG MC, PAPSIDERO LD, et al. Quantitation of prostate-specific antigen in serum by a sensitive enzyme immunoassay. Cancer Res 1980; 40:4658-4662.

(*14*) WHITMORE WF JR. The rationale and results of ablative surgery for prostatic cancer. Cancer 1963; 16:1119-1132.

(*15*) KELSOE GH, WELLER TH. Immunodiagnosis of infection with *Schistosoma mansoni:* Enzyme-linked immunosorbent assay for detection of antibody to circulating antigen. Proc Natl Acad Sci USA 1978; 75:5715-5717.

(*16*) CHU TM, WANG MC, VALENZUELA LA, MERRIN C, MURPHY GP. Isoenzymes of prostatic acid phosphatase. Oncology 1978; 35: 198-200.

(*17*) WANG MC, KURIYAMA M, LOOR RM, LI SC, CHU TM. Biochemical characterization of human prostate-specific antigen. Fed Proc 1981; 40:1564.

(*18*) FOTI AG, COOPER JF, HERSCHMAN H, MALBAEZ RR. Detection of prostate cancer by solid phase radioimmunoassay of serum prostatic acid phosphatase. N Engl J Med 1977; 297:1357-1359.

(*19*) WATSON RA, TANG DB. The predictive value of prostatic acid phosphatase as a screening test for prostatic cancer. N Engl J Med 1980; 303:497-499.

(20) LINDHOLM GR, STIRTON MS, LIEDTKE RJ, BATJER JD. Prostatic acid phosphatase by radioimmunoassay. JAMA 1980; 244: 2071-2073.
(21) KILLIAN CS, VARGAS FP, SLACK NH, MURPHY GP, CHU TM. Prostatic-specific acid phosphatase versus acid phosphatase in monitoring patients with prostate cancer. Clin Chem 1981; 27: 1064.
(22) KURIYAMA M, WANG MC, LEE CL, et al. Use of human prostate-specific antigen in monitoring prostate cancer. Cancer Res 1981; 41:3874-3876.